Some Aspects of the Molecular Pathology of Breast Cancer

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DECLARATION

I declare that the work incorporated in this thesis has been performed by myself, except as stated in the Acknowledgements, and that the thesis was composed by myself. Histopathological characterisation of cancer type and grade was performed by Dr T.J. Anderson. DNA ploidy analysis was performed by Ms C Doris. Dr R.A Hawkins provided oestrogen receptor concentrations. Dr A.M. Thompson performed c-erb B2 mRNA analysis.

March 1995

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THE UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS

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This thesis examines some aspects of the molecular pathology of breast cancer. Disregulation of the c-erb B2 oncogene was related to several histopathological and biochemical features of breast cancer. Other genes, specifically THRA1 and urokinase plasminogen activator were investigated for disregulation and possible interaction with c-erb B2 in the biology of breast cancer.

A differential PCR technique for the detection of c-erb B2 gene amplification, was tested and validated for use on paraffin embedded methacarn fixed breast cancer tissues. Tissues from 314 breast cancers and 43 control samples were assessed for c-erb B2 gene amplification by dPCR. In clinical material the results were not affected by the DNA contribution of normal tissue elements or by cancer DNA ploidy change. C-erb B2 gene amplification was detected in 55% of invasive cancers and in 66% of *in situ* cancers. C-erb B2 protein overexpression in breast cancer cells, as determined by specific immunohistochemistry, was only detected in 11% of invasive cancers and 43% of *in situ* cancers. Comparisons show that a substantial number of cancers with c-erb B2 amplification lack detectable protein overexpression. The nature of c-erb B2 gene disregulation in breast cancer appears to be complex and multiple combinations of biological events are suggested.

Detailed comparison of c-erb B2 gene amplification and overexpression with biochemical and histopathological features was made of sets of breast cancers which formed part of a breast screening study. C-erb B2 gene amplification did not show association with any of the features assessed, such as cancer size, histopathological type or grade, lymph node status , DNA ploidy status, or oestrogen receptor status. C-erb B2 expression was not associated with cancer size, lymph node status or DNA ploidy status, but positive associations were found with low concentrations of oestrogen receptors, high cancer grade, and cancer histological type; the association with high cancer grade and cancer type were not independent of oestrogen status. The possibility that oestrogen receptor has a functional relationship with c-erb B2 resulting in downregulation of c-erb B2 expression is discussed. The frequency of c-erb B2 disregulation, either amplification or overexpression, was similar in each of the screening groups (newly, previously and never screened) and indicated that it could be an early event.

A possible allelic imbalance and pattern of allele amplification of c-erb B2 were investigated in 70 breast cancers and 34 control DNAs. A 1.1kb sequence spanning a restriction fragment length polymorphism was PCR amplified and digested with restriction enzymes *MboI* and *PvuII*, to reveal one or two alleles. The distribution and frequencies for c-erb B2 amplified, c-erb B2 non-amplified and control DNA were tested for goodness of fit with the Hardy-Weinberg equilibrium. The genotype distribution from c- erb B2 amplified cancers differed significantly from non- amplified cancers. In heterozygous cancer uneven allele band intensity was observed in 58% of amplified, 31% of non-amplified. More than one mechanism of gene amplification may occur in the development and progression of breast cancer.

THRA1, a nuclear receptor gene frequently co-amplified with c-erb B2, was examined for functional mutations within exons which code for the zinc finger binding regions of the receptor. SSCP analysis in 90 breast cancers and 24 controls identified a deviant banding pattern in 5 cancers and 2 controls, which resulted from a C to T transition in exon 5. This base change did not alter the amino acid coding sequence of the gene, therefore the specificity of THRA1 remains unaltered in this series of breast cancers.

The urokinase plasminogen activator gene was examined for overexpression and for gene amplification in 134 breast cancers. Using dPCR techniques, gene amplification was not detected in any breast cancers. Overexpression of uPA was detected in 35% of invasive cancers, and in 19% of *in situ* cancers, and was not associated with other markers of cancer aggression, such as lymph node status, or histopathological grade. There was a positive correlation between cancers which overexpressed c-erb B2 and uPA. This could indicate either a functional relationship between these proteins or a non-specific increase in cellular protein expression.

A model for the involvement of c-erb B2 in breast cancer progression is proposed. Mechanisms of c-erb B2 gene amplification and interaction with other cellular proteins are discussed.

CHAPTER 1

Literature Review

1.1 Importance of Breast Cancer.

Breast cancer has long been recognised as an important disease seriously affecting the health of women and men. The Chambers Encyclopaedia of 1751 described it as "a most dread disease, particularly of the celibate and barren". In this century, studies of risk factors, causative agents and molecular biology of the disease has increased our knowledge of the initiation, development and progression of breast cancer. One overall aim is to treat cancer effectively and ultimately to prevent the occurrence of cancer. A means of getting closer to this aim is to identify the genetic events which occur in breast cancer and understand their biological consequences.

Recent surveys show that 1 in 9 women in the United States of America (American Cancer Society 1991), and 1 in 14 women in Scotland will develop breast cancer (Scottish Breast Cancer Screening Program Report 1993), and it will be responsible for the death of 25% of these women (Sondik 1994). The incidence of breast cancer has probably increased during this century, and is most noticeable in women up to 50 years old (Kelsey and Berkowitz 1988, Ranstam et al 1990). The majority of cancers occur in women aged over 50 years; incidence in this group may have increased due to an increased life expectancy (Sondik 1994). This suggests that an accumulation of genetic events over time may be necessary for the development of non- familial breast cancer (Armitage and Doll 1954, Peto et al 1975). At a macroscopic level the cumulative effect of these genetic disregulatory events is reflected phenotypically by the overall histopathology of breast cancer.

1.2 Histology and Pathology of the Breast.

Breast cancer presents clinically as a heterogeneous disease and is classified by histopathological type, and other features such as cancer grade and biological markers (discussed below). Breast cancers can present either symptomatically or from routine mammographic screening.

1.2.1 The Structure of the Normal Breast.

The normal breast consists of a branching network of ducts, formed by epithelial cells, which originate at the lobules and terminate at the nipple. Lobular units produce milk during lactation and are surrounded by fatty cells. Milk is secreted into the ducts and channelled to the nipple. Epithelial cells in the non-lactating breast are largely under the influence of progesterone and oestrogen, and undergo monthly changes of proliferation and apoptosis (Page and Anderson 1987).

1.2.2 Breast Cancer.

Detection of a breast lump commonly leads to a biopsy for pathological investigation and determination of oestrogen receptor status. In addition many cancers are detected by routine mammographic screening, such as in the Edinburgh Randomised Breast Screening Project. Mammographic abnormalities indicating breast cancer may be detected in 2.2% of women at the primary screen (prevalence screen) (Anderson et al 1991). The prevalence screen may include some cancers which are clinically symptomatic and some non-palpable lesions. Subsequent screens (incidence screen) may detect early lesions and cancers which arise between screens are true "interval" cancers, which may include groups of cancers representing early and or rapidly growing cancers. Cancers detected at screening are important in the investigation of

molecular lesions, particularly when attempting to establish a sequence of events in cancer progression. Benign lesions of the breast include atypical ductal hyperplasia, radial scars and fibroadenoma. Each can cause disturbance to the tissue architecture due to cellular proliferation. Transformation steps between benign and malignant lesions have not been clearly identified (Bodian 1993). Breast cancer is sub classified into several histopathological types (reviewed in Page and Anderson 1987). *In situ* cancers are classified according to the presence and type of neoplastic cells within the ducts, without infiltration into the surrounding stroma. *In situ* cancers can be either ductal, lobular type, or Pagets disease of the nipple. True invasive breast cancer is frequently ductal carcinoma of no special type. Special types of invasive cancer include lobular, tubular, medullary or mucinous cancers, and are characterised by particular patterns of growth. Invasive cancers often also have an *in situ* component. Classification is not always unequivocal and cancers of mixed type occur. The cancers can be further classified by grading, using the criteria of gland formation, nuclear pleomorphism, and frequency of mitosis (Elston 1987). Higher cancer grades signify increasing aggressiveness (Tubiana and Koscielny 1991).

1.3 Initiating Events of Breast Cancer.

1.3.1 Environmental Factors.

The contribution of environmental factors in the aetiology of breast cancer is unclear. Environmental mutagens, cigarette smoking, and diet have all been implicated in increased risk (Hunter and Willet 1993, Kelsey and Horn-Ross 1993, Palmer and Rosenberg 1993). In addition reproductive history and hormonal influences in individual patients can also contribute towards increased risk. These factors can apply in tandem and present a complex pattern from which to assess individual risk.

The salient points from several review articles are discussed below (Kelsey and Horn-Ross 1993, Hunter and Willet 1993, Palmer and Rosenberg 1993).

1.3.2 Diet and Cancer Risk.

High fat diets have been suggested as a risk factor in breast cancer. High fat diets may increase the incidence of mammary tumours in rodents (Tannenbaum 1942), and differences in the frequency of breast cancer between countries and continents have suggested that dietary factors which apply in a general fashion throughout a population may play a role in the development of the disease in man (Kelsey and Horn-Ross 1993). The increased frequency of breast cancer in Japan from 1900 to the present has been linked to the Japanese diet becoming westernised (Hunter and Willet 1993). In addition, Americans of Japanese origin, residing in the United States of America, have a similar frequency of breast cancer to Americans of European origin (Haensel and Kuntasa 1968). However this association may be weak as reproductive or hormone status may also have changed in these populations (Bernstein and Ross 1993). Conducting properly controlled trials for the effects of diet on human cancer initiation has proved difficult.

1.3.3 Environmental Mutagens and Cancer Risk.

The impact of environmental mutagens on the development of breast cancer is not known. Epidemiological studies of the effects of compounds such as tobacco smoke, alcohol, ionising radiation, and pesticides on the incidence of breast cancer have failed to find any strong associations (Adami et al 1990, John and Kelsey 1993). For example, tobacco smoking is thought to only marginally increase breast cancer risk (O'Connell et al 1987); indeed one study indicated a weak protective effect (Baron 1994).

Animal models have been used to test chemical mutagens *in vivo*. Mutagens, such as N-nitro-N methyl urea have induced the development of tumours when injected directly into rodent mammary fat pads (Sukumar et al 1986, Zarbl et al 1987). These studies indicate a potential role for chemical mutagens, but extrapolation to human breast cancer is complicated by interspecies variability and incomplete knowledge of mutagen biochemistry.

1.3.4 Steroid Hormones and Cancer Risk.

Oestrogens and progestogens play an important role in the normal development and functions of the breast (Anderson and Battersby 1989, King 1993). The biology and disregulation of oestrogen and oestrogen receptors is discussed below (section 1.11). Increased risk of breast cancer has been associated with early age of menarche, late menopause, nulliparity and full term pregnancy at an older age (Kelsey et al 1993). An early age for a first child and performance of breast feeding are thought to have some protective effect (MacMahon et al 1970). These effects are thought to be due to the influence of steroid hormones on the breast cells (Byers et al 1985), particularly oestrogen and progesterone. The incidence of breast cancer in men is only 0.01X that of women, although cancers in men are morphologically identical (Thomas 1993); they may be associated with rare abnormalities of the androgen receptor (Trapman and Brinkman 1993, Wooster et al 1992).

1.4 Genetic Factors in Breast Cancer.

For environmental or hormonal factors to influence cellular growth characteristics they must in some way disturb normal genetically controlled cellular mechanisms. These genetic control mechanisms may also be affected by mutation or disregulation which may not have an environmental basis. Genetic factors can be categorised into two main types; those which are inherited and occur constitutionally; and mutations or disregulations which occur in somatic cells. This section will outline different kinds of genetic disregulation and focus on a subset of these.

1.4.1 Inherited Genetic Lesions.

A familial element has been identified in many early onset breast cancers, but it has been estimated that familial cancers only represent 4-9% of breast cancers (Houlston et al 1992). Familial cancers are characterised by an autosomal dominant pattern of inheritance. They can be bilateral, show multifocality and can be associated with other cancers such as ovarian cancer (Adami et al 1990). Intensive studies are currently underway to identify the genes involved. One system of germline mutations involves the p53 tumour suppressor gene, originally identified in families with multiple cancer types (Li and Fraumeni 1969) and is discussed in section 1.7.3. Two genes, BRCA1 and BRCA2 have been identified as candidate genes by linkage analysis in breast cancer families (Futreal et al 1992, Hall et al 1990b, Wooster et al 1994). BRCA1 has recently been identified as a potential transcription factor, and located to 17q12-21 (Miki et al 1994). Several mutations, which result in protein truncations, missense mutations and altered splice sites, have been detected in BRCA1 in families with breast and ovarian cancer (Freidman et al 1994, Simard et al 1994, Castilla et al 1994). BRCA2 is genetically linked to markers on chromosome

3q12-13, however its precise location and the function of the gene remain unknown (Wooster et al 1994). These two genes may account for two thirds of familial breast cancers, or about 5% of all breast cancers (Ponder 1994), therefore other key genes remain to be found. The extent of involvement of these genes in sporadic cancers is not yet known. Linkage analysis of genes and markers on the long arm of chromosome 17 have indicated that there may be another breast cancer associated gene located in this region (Easton et al 1993, Narod et al 1991).

1.4.2 Non-familial Breast Cancer.

The majority of breast cancers are sporadic and occur in women aged over 50, and are not associated with a family history of cancer (Kelsey et al 1993). This suggests that these breast cancers may arise from an accumulation of disruptive somatic molecular events over time, which may include those genes involved in hereditary cancer.

The number of genetic events required for the transformed phenotype may be variable, and range from a theoretical one, to two (Armitage and Doll 1957, Knudson 1989), to many (Fearon and Vogelstein 1990, Thompson et al 1992). In contrast to breast cancer, a clear sequence of genetic events has been identified in colon cancer, and associated with particular pathological stages of cancer progression (Fearon and Vogelstein 1990). The breast presents a more complex pathological picture as precursor lesions have not been identified (Bodian 1993). The variety of pathological types may suggest that different genetic disregulatory pathways or different sequences of events may be involved, which may share common events. Proto-oncogenes and tumour suppressor genes have been implicated as targets for molecular disregulation (Bishop 1991) and may play an important role in breast carcinogenesis and progression; they are discussed below.

1.5 Mechanisms of Gene Disregulation.

1.5.1 Chromosomes.

Gross chromosomal disruption is evident in some breast cancer cell lines (Ali et al 1988a, Sasi et al 1991, Sato et al 1991), and is also evident in cell material from surgical specimens of breast cancer (Gebhart et al 1986). Chromosomal disregulation can be represented by translocations (either balanced or unbalanced), dicentric chromosomes, acentric chromosomes, double minutes, deletions, duplications and complete or partial aneusomy. These abnormalities are usually detected by staining metaphase chromosome spreads prepared from actively dividing cells. *In vivo* metaphase spreads are difficult to obtain. Extrapolation of *in vitro* chromosomal disregulation in cell lines to *in vivo* cancers may not be a valid comparison, as cancer cells which grow in culture may represent a biased sample of the whole population. In addition cells in culture show clonal expansion during culture (Meltzer et al 1991), indicating that the karyotype of established cell lines may not accurately reflect the chromosomal status of the original breast cancer. The transformed phenotype of cancer cells as a result or resulting in gross chromosome disruption is likely to be due to the action(s) of specific gene(s), which are disregulated by either a physical disturbance (direct gene disfunction) or a regulatory disturbance (indirect gene disfunction).

1.6 Direct Gene Disfunction.

Direct gene disfunction can arise from a physical alteration of the basic nucleic acid sequence of the gene or its promoter regions. This could take the form of: point mutations, variable sized deletions or insertions, translocations or amplifications.

Each of these has the potential to abrogate or increase transcription, or alter the amino acid sequence of the gene product.

1.6.1 Normal Gene Function.

The basic structure of a typical gene is shown in Figure 1, and consists of a non-transcribed promoter region, and a transcribed region consisting of introns and exons. Gene transcription results in the formation of a messenger RNA (mRNA), which is enzymatically spliced in the nucleus to form a functional mature mRNA, consisting only of exons. Gene translation takes place in the cytoplasm, on ribosomes attached to the endoplasmic reticulum. Amino acids bound to transfer RNA (tRNA) bind specifically, according to the triplet code, to the mRNA located on the ribosome. The protein is formed when peptide bonds are synthesised enzymatically between adjacent amino acids. Post-translational processes complete the three dimensional structure of the protein. The promoter region of a gene plays a key role in the initiation of transcription and can be regulated by promoter, enhancer or gene suppressor proteins. Sequences within the promoter which are recognised by these proteins are called *cis*-acting sequences, and the binding regions of the proteins are termed *trans*-acting elements. Several sites of initiation can be present within each promoter region, and includes common sequences such as CAAT and TATA. Genes may also have enhancer elements, a sequence which is orientation independent and can increase transcription even when located up to several kilobases away. Enhancer elements can also be located within introns. Suppressor proteins are described below.

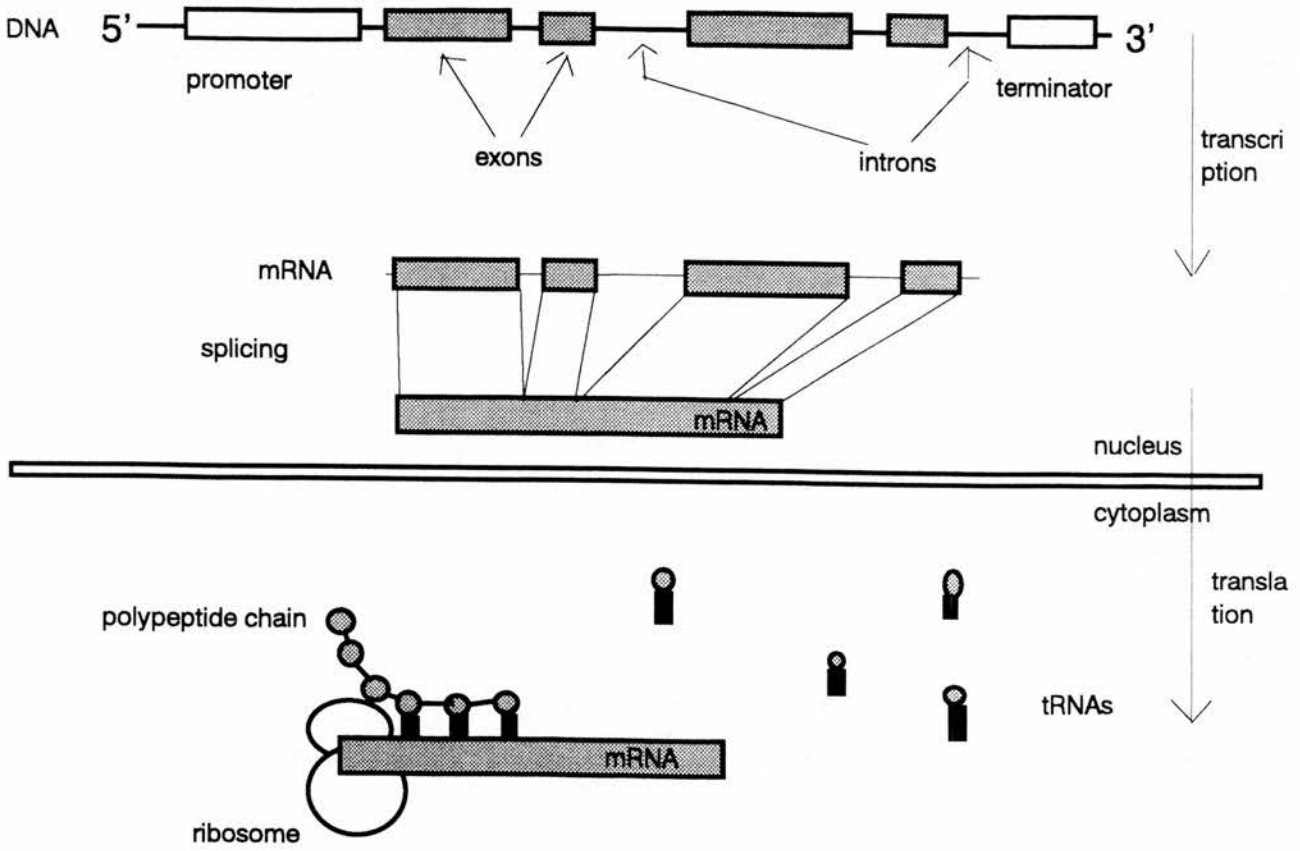
1.6.2 Chromosome Translocation.

Translocations are frequent events in cancer tissue cells (reviewed by Cleary 1991) and can be influential in the development of some cancers, such as in chronic myeloid

Figure 1.

Ideogram of gene transcription and gene translation. Gene translation takes place within the nucleus of the cell. Single stranded mRNA is transcribed from double stranded DNA consisting of introns and exons, followed by gene splicing to form a mRNA consisting only of exons. mRNA enters the cytoplasm and attaches to a ribosome located on the endoplasmic reticulum. Amino acids bound to tRNA bind in a sequence specific manner to mRNA, where adjacent amino acids bind to form a polypeptide chain.

Figure 1


DNA
5'
3'
promoter
terminator
exons
introns
transcri
ption
mRNA
splicing
mRNA
nucleus
cytoplasm
transla
tion
polypeptide chain
tRNAs
mRNA
ribosome

leukaemia (deKlein et al 1982) which is associated with the Philadelphia chromosome (t9:22, [q34,q11]). Chromosome analysis of breast cancer cell cultures has identified many structural and numerical chromosome alterations, including DNA deletions and translocations (Dutrillaux et al 1990, Ferti-Passantonopoulou et al 1987). Some chromosomal abnormalities may be primary or secondary events in the oncogenic process, however others may merely reflect non-specific chromosomal disregulation as a result of other primary events. The difficulties involved in assessing the karyotype in solid tumours, as discussed above, suggests that molecular techniques may be a more universal method of determining chromosomal damage.

1.6.3 Loss of Heterozygosity.

DNA deletion is another common form of chromosomal damage. Examining loss of heterozygosity has proved an effective method of identifying candidate DNA sequences of relevance to cancer development. Most genes are present as one of two or more alternative forms, and are termed alleles. Polymorphisms between alleles can be detected by various molecular methods, and when present are classified as homozygous (both alleles being the same) or heterozygous (alleles are different). Where individuals are heterozygous at a particular locus, comparison of normal and cancer DNA can detect loss of one allele. Loss of heterozygosity is widely observed in cancer DNA and is frequently detected via anonymous DNA sequences. The real significance of loss is usually unclear until further exploration of the DNA region reveals candidate genes.

Loss of heterozygosity has been observed at a large number of loci in breast cancers and has identified regions of chromosomes 1, 3, 6, 11 16, and 17 which may contain genes important to the development and progression of breast cancer (Borg et al

1992, Devilee et al 1989, Futreal et al 1992, Lundberg et al 1987). Regions frequently lost may indicate the presence of a tumour suppressor gene. Allele loss at 17q leading to the presence of hemizygosity has previously been reported in 60% of breast cancers (Cropp et al 1990) however loss at any single locus is a less frequent event (Devilee et al 1991, Sato et al 1991).

1.6.4 Gene Amplification.

In other animals than man, gene amplification (an increase in the number of gene copies) is a developmentally programmed event which allows high levels of expression of a particular gene at a specific time (Stark et al 1989). In man, gene expression is controlled by promoters or silencers which effect transcription (Marshall 1991), and gene amplification does not normally take part in this process. In cancer cells however, genes can be amplified in an oncogenic process, either by changes in cell DNA ploidy or by specific gene amplification.

The mechanisms by which genes amplify in man are not clearly understood, however some regions of chromosomes are prone to amplification (Lammie and Peters 1991). Amplified regions of DNA can extend from a few hundred to thousands of kilo base pairs (Stark et al 1989). Large amplifications can sometimes be seen by light microscopy as either extended chromosomal regions, termed homogeneously stained regions, or extra chromosomal elements, usually called double minutes (Stark et al 1989). Genes can be amplified as the result of physico-chemical alterations to cells, such as hydroxyurea, hypoxia, ultraviolet and ionising radiation (Schimke 1988, Stark et al 1989). Gene amplifications are not always stable and gene loss, including regions of amplification, can occur in the absence of selection pressure (Saito et al 1989).

Several models of gene amplification have been proposed, and categorised into replication driven and segregation driven models. Replication driven models include : i/ Onion skin model - head to head or head to tail tandem arrays of amplified units created by independent recombination events, Figure 2 (Stark et al 1989); ii/ Extrachromosomal double rolling circle model- a single round of local DNA replication forming a circle containing an inverted duplication which is excised from the chromosome by double recombination and then amplified as a double rolling circle, Figure 3 (Passananti et al 1987); iii/ Chromosomal spiral model- an inverted duplication is formed when replication switches strands, Figure 4 (Nabaltonglu and Meuth 1986). Segregation driven models include : i/ Deletion plus episome model- a circular sequence created by either a recombination across a replication loop which contains a functional replication origin or by re-replication. The episomes formed are acentromeric and will therefore segregate randomly at mitotic cell divisions (Schimke et al 1986) ; ii/ Sister chromatid exchange model- sister chromatids recombine unequally before mitosis to form head to tail joining of amplified domains, forming an extra long chromosome arm with amplified gene copies separated by long stretches of co-amplified material, Figure 5 (Guilotto et al 1986).

1.7 Tumour Suppressor Genes.

Apparent changes in the activity of specific genes may not involve physical changes to the coding or promoter sequence of a gene. Alterations in the rate of protein production may be due to abnormal levels of promoters, enhancers or suppressors (Marshall 1991, Spandidos 1985), proteins which effectively switch on or off gene transcription. These genes are subject to disregulation in some cancers and are usually detected by loss of heterozygosity analysis (see section 1.6.3). Tumour suppressor genes can regulate cell growth in a negative fashion by blocking

Figure 2.

A model for amplification of mammalian DNA involving unscheduled DNA synthesis plus recombination. The drawing is from Stark and Wahl (1984). Bi-directional replication at an origin generates a bubble that can undergo further rounds of unscheduled DNA replication, resulting in a nested set of partially replicated duplexes. There are only two contiguous chromosomal strands. It is possible for linear duplex DNA to become detached from the structure if two replication forks can approach one another very closely (pathway 3). Recombination within the same duplex could generate extra chromosomal circles (pathway 2), while multiple recombinations among different duplexes could resolve the structure into an intrachromosomal linear array (pathway 1).

Figure 2

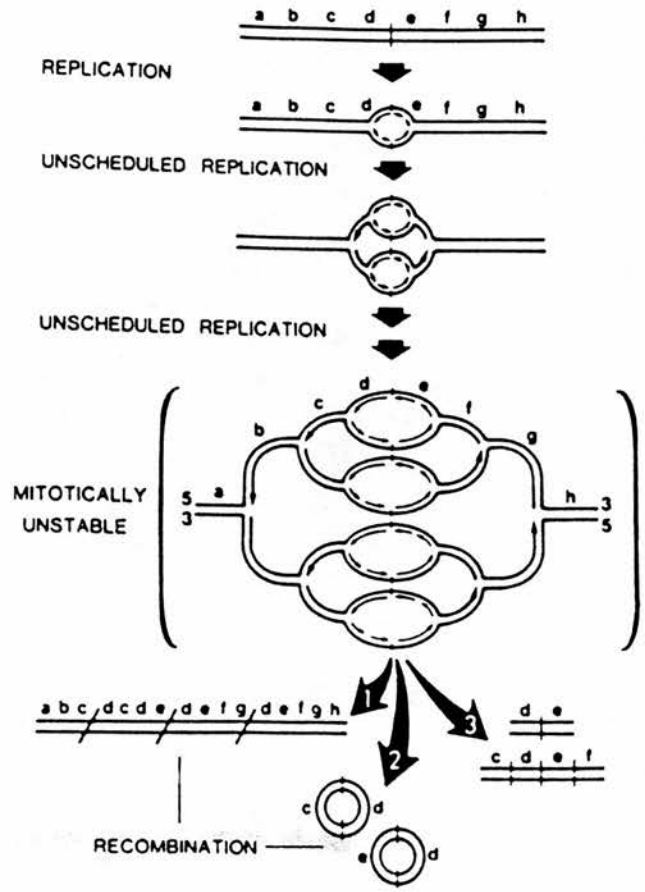

a b c d • f g h
REPLICATION
a b c d • f g h
UNSCHEDULED REPLICATION
UNSCHEDULED REPLICATION
MITOTICALLY
UNSTABLE
5
3
a
b
c
d
•
f
g
h
3
5
a b c d c d • d • f g d • f g h
1
2
3
d •
c d • f
c
d
•
d
RECOMBINATION

Figure 3.

A model for amplification of mammalian DNA involving inverted duplications. The drawing is from Stark and Wahl (1989). A circular model containing an inverted duplication (rectangles) harbouring the gene to be amplified (dots) is excised from the chromosome, yielding also a chromosomal deletion (not shown). After the inverted sequence is replicated once, homologous recombination takes place between a copy of the newly replicated sequence and the copy yet to be replicated. This results in a flip of the replicating forks (small arrows) so that they are now chasing each other. Further replication leads to amplified arrays of inverted duplications derived from a single initiation of DNA replication during a single S phase.

Figure 3

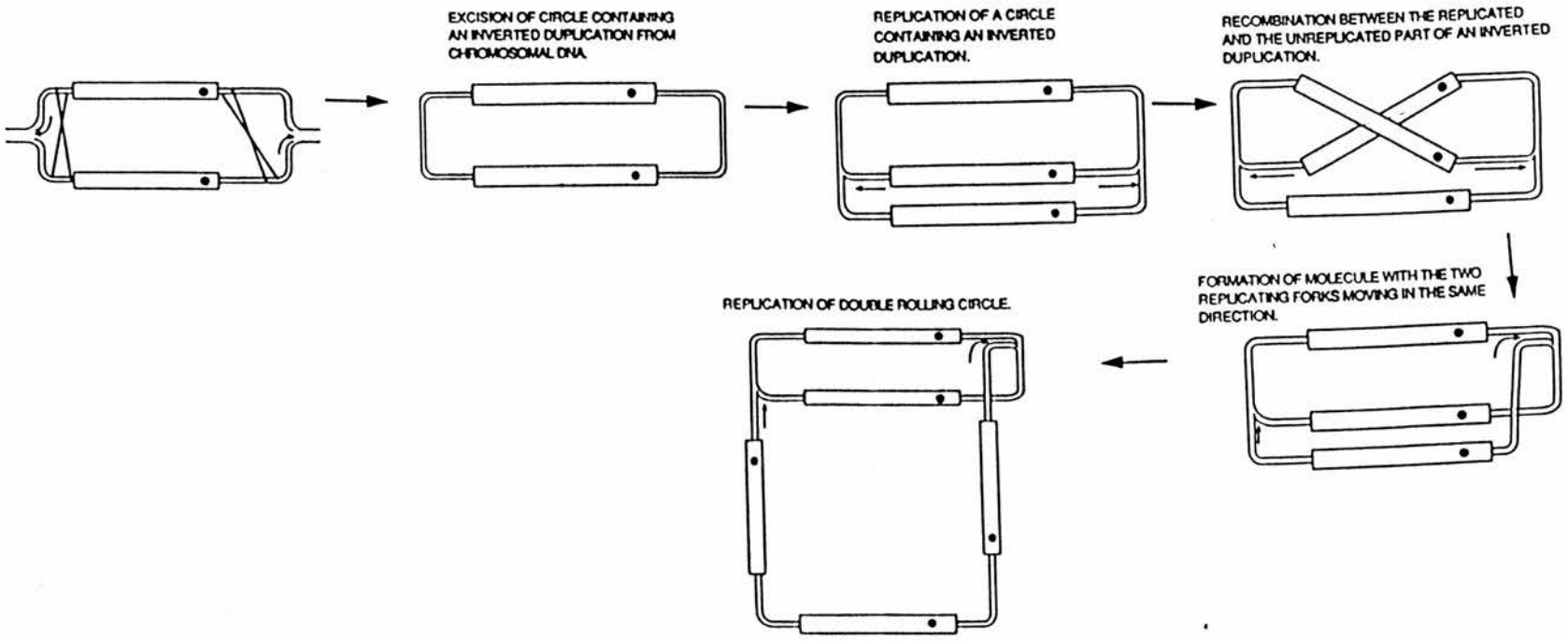

EXCISION OF CIRCLE CONTAINING AN INVERTED DUPLICATION FROM CHROMOSOMAL DNA.
REPLICATION OF A CIRCLE CONTAINING AN INVERTED DUPLICATION.
RECOMBINATION BETWEEN THE REPLICATED AND THE UNREPLICATED PART OF AN INVERTED DUPLICATION.
FORMATION OF MOLECULE WITH THE TWO REPLICATING FORKS MOVING IN THE SAME DIRECTION.
REPLICATION OF DOUBLE ROLLING CIRCLE.

Figure 4.

A model for inverted duplication formation and intrachromosomal amplification. The drawing is from Stark and Wahl (1989). (a-d) Formation of a head to head (C'/BC) joint by copy-choice recombination. (d') Structure obtained if a head-to head (C'/BC) joint and a tail-to-tail (G/H'G') joint are formed by similar events at both ends of the replication bubble. (e) Amplification of the resulting inverted duplication by intrachromosomal double rolling circle replication.

Figure 4

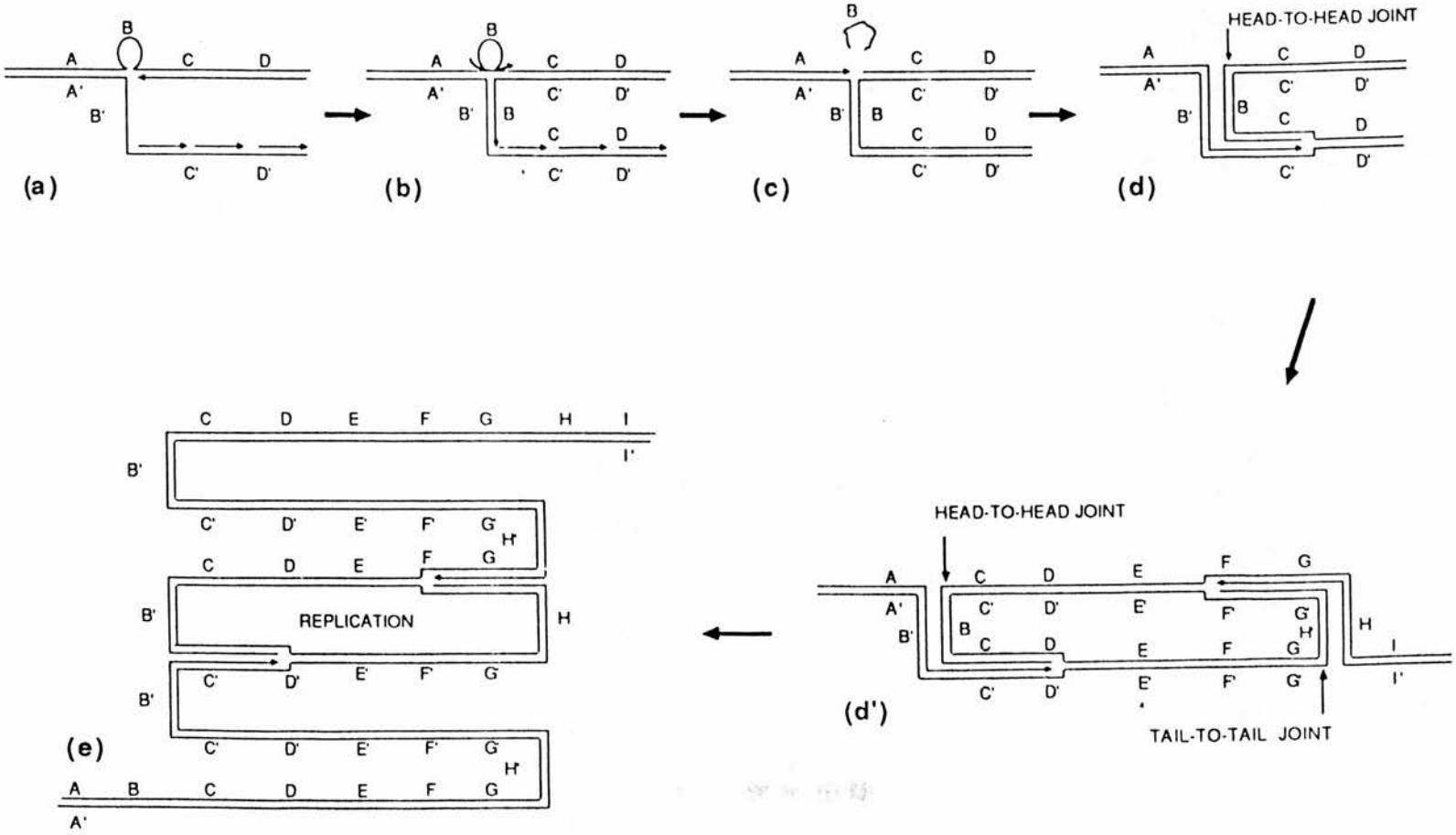
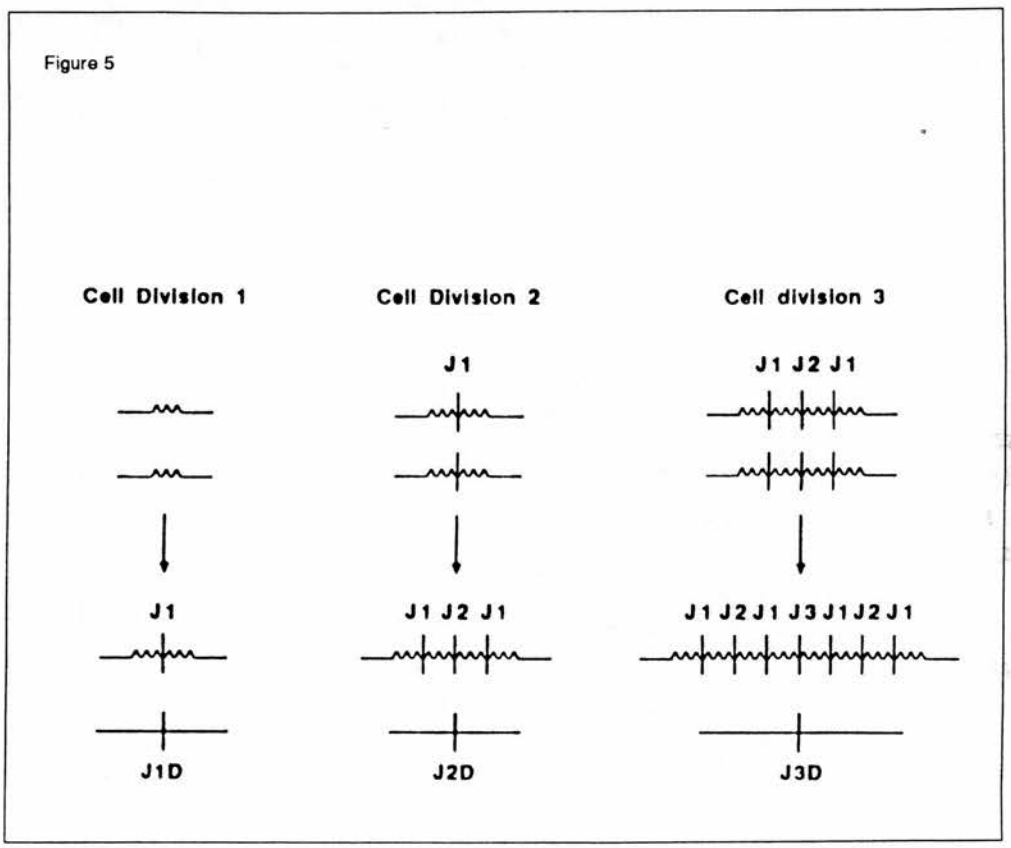

(a)
(b)
(c)
(d)
HEAD-TO-HEAD JOINT
(d')
HEAD-TO-HEAD JOINT
TAIL-TO-TAIL JOINT
REPLICATION
(e)

Figure 5.

A model for gene amplification in which sister chromatids recombine unequally before mitosis. A lineage is shown in which copy number increases maximally at each cell division. Note that only one pair of sister chromatids is shown and that each daughter cell still has an unaltered chromosome. After the third step there are four copies of J1 and two copies of J2 but only one copy of J3. J2 might be a single copy number if the increase were not maximal at step 3. The model predicts formation of both single copy and amplified novel joints in head-to-tail arrangements.

Figure 5


Cell Division 1
Cell Division 2
Cell division 3
J1
J1 J2 J1
J1
J1 J2 J1
J1 J2 J1 J3 J1 J2 J1
J1D
J2D
J3D

transcription of genes or by involvement in cell cycle control (Marshall 1991). Few tumour suppressors have been extensively studied in breast cancer, the notable exceptions being p53 and the retinoblastoma gene (Rb). It is yet to be resolved whether familial breast cancer genes BRCA1 and BRCA2 can act as tumour suppressor genes. The loss of tumour suppressor action, can occur in a "two hit" process, in which the first "hit" may take the form of an inherited disregulation in one allele and the second "hit" occurs somatically to knock out the remaining allele, resulting in complete inactivation of both copies (Knudson 1989). This is known to occur in both the Rb gene in familial retinoblastoma (Cavanee et al 1983), and in p53 in colon cancer (Van Den Berg et al 1989) and is likely to result in cell proliferation (Hollstein et al 1991, Nigro et al 1989) contributing to a transformed phenotype. The involvement of these and other, as yet unidentified, tumour suppressor genes in breast cancer oncogenesis is indicated by the frequent loss of heterozygosity at specific chromosome locations, such as 1p and 1q (Borg et al 1992), 6q (Devilee et al 1991), 11p and 13q (Devilee et al 1989), 13p (Lundberg et al 1987), 16q, (Sato et al 1990), and 17q (Futreal et al 1992). Candidate genes have not yet been identified at all of these loci.

1.7.1 Retinoblastoma Gene.

The retinoblastoma gene (Rb) plays a critical role in the development of retinoblastoma (Knudson 1971). It has been shown that Rb protein acts to halt progression through G1 phase of the cell cycle (Goodrich et al 1991) and the biological consequence of loss of Rb function may be unscheduled cell proliferation. Mutations which abrogate Rb function have been found in a wide variety of cancers, including breast cancer (Friend et al 1986, Lee et al 1988, T'Ang et al 1988). Loss of heterozygosity at chromosome 13q14, the chromosomal location of the Rb gene, has been observed in 21-29% of breast cancers (Devilee et al 1989). Structural

abnormalities in the Rb gene have been found in 19% of breast cancers (Varley et al 1989), suggesting that mutations in Rb may contribute to the malignant phenotype in some breast cancers. Loss of Rb function has been found to correlate with more advanced and less differentiated breast cancers (Varley et al 1989). However, other important breast cancer genes may be located close to the Rb locus, such as BRCA2 (Wooster et al 1994), perhaps indicating that the role of the Rb gene is not fully determined.

1.7.2 p53 tumour Suppressor Gene.

Mutations in p53 contribute to the development of up to 50% of all human cancers (Marx 1993), and are present in approximately 50% of breast cancers (Cattoretti et al 1988, Osborne et al 1991). Recent studies have shown that the p53 protein transactivates other genes (CIP1/WAF1) whose products inhibit the cyclic kinases required to drive cells through the cell cycle (Harper et al 1993, El-Deiry et al 1993). Allele loss in breast cancer has frequently been observed on the short arm of chromosome 17, and 17p13, a location where the p53 gene resides (Coles 1990, Devilee et al 1990). Mutations in p53 can occur in 25-40% of sporadic breast cancers (Lemoine et al 1994) and is associated with an aggressive cancer phenotype and poor prognosis. Its role in breast cancer progression remains unclear.

1.8 Oncogenes.

1.8.1 Introduction.

Oncogenes and protooncogenes are a large family of genes which, unlike tumour suppressor genes which can switch off gene transcription, can switch on or increase transcription of target genes (Rochlitz and Benz 1989). Oncogenes were originally

identified as the transforming sequences of acutely transforming retroviruses (usually prefixed v-), which were subsequently shown to be mutated forms of host genes (usually prefixed c-) which had been transduced from the normal host genome by retroviruses (Bishop 1991).

1.8.2 Oncogene Function.

Most oncogenes have a normal counterpart within the mammalian genome, the exceptions being some oncogenes of DNA viruses such as E1A, and E6 (Shenk and Flint 1991, Laimins 1993). Oncogenes can be classified into four main categories of protein: growth factors; growth factor receptors; transducers of growth factor responses; and transcription factors (reviewed in Hunter 1991). Most oncogenes are involved in the cascade of events by which growth factors stimulate cell division (Cantley et al 1991), and it is only when they are inappropriately activated that they can take part in the oncogenic process (Hunter 1991).

1.8.3 Oncogene Activation.

The activation of protooncogenes can occur in different ways. Activated oncogenes implicated in human breast cancer include: amplification of c-myc, L-myc, N-myc, c-int-2 and c-erb B2; rearrangements of L-myc, N-myc, c-myb, c-mos and Ha-ras; allelic loss of Ha-ras; and amplification or mutation of Ki-ras, Ha-ras and N-ras (Cline et al 1986, Lidereau et al 1988, Rochlitz and Benz 1989). Many of these abnormalities are present at low frequencies in breast cancer populations, with amplifications of c- myc and c-erb B2 and allelic deletions of Ha-ras and c-myb being most prevalent (Rochlitz and Benz 1989, Callahan 1989). The oncogenic potential of nuclear receptors has also been recognised (Hunter 1991), and disregulation of members of this family may be candidates for involvement in breast cancer, both

oestrogen receptor and thyroid hormone receptor (THRA1) are discussed in sections 1.11 and 1.12.

Oncogenes and tumour suppressor genes are scattered throughout the genome, see Figure 6. Several genes of potential importance in the development of breast cancer are located on chromosome 17, see Figure 7, and include the p53 tumour suppressor gene on the p arm, and BRCA1, c-erb B2 and THRA1 on the q arm. This thesis focuses on detailed examination of chromosome 17q, and on c-erb B2 and THRA1, partly because c-erb B2 was initially associated with poor prognosis in breast cancer (Slamon et al 1987) and THRA1 which can be co-amplified with c-erb B2 (Tavassoli et al 1989, Tsuda et al 1989).

1.9 C-erb B2.

1.9.1 Introduction.

C-erb B2 is a member of a growing family of tyrosine kinase receptor genes, which includes c-erb B1 (the epidermal growth factor receptor gene), c-erb B3 and c-erb B4 (which have as yet unknown functions).

1.9.2 Oncogenic Potential of c-erb B2

C-erb B2 was first identified as a potential oncogene in rodents with neuroglioblastoma (hence called neu) where a single point mutation in the transmembrane region of the protein was identified as having transforming abilities (Bargmann et al 1982). The single point mutation may result in a constant activation signal transmitted to the cytoplasm (Shih et al 1981). In later studies of transgenic

Figure 6.

Ideogram of human metaphase chromosomes illustrating the approximate locations of some oncogenes and suppressor genes. A more detailed map of chromosome 17 (within box) is illustrated in Figure 7. Genes illustrated are;

c-abl1	Abelson murine leukaemia viral oncogene homolog .
c-abl2	Abelson murine leukaemia viral oncogene homolog 2.
c-akt	murine thyoma virus oncogene homolog.
BCL1	B cell CLL/lymphoma 1.
BCL2	B cell CLL/lymphoma 2.
c-blym1	avian lymphoma virus derived transforming sequence.
DCC	deleted in colorectal cancer.
c-elk1	member of ETS oncogene family.
c-elk2	member of ETS oncogene family.
c-erb B1	(EGFR) epidermal growth factor receptor, avian erythroblastic leukaemia viral oncogene homolog.
c-erb B2	avian erythroblastic viral oncogene homolog 2.
c-erg	avian erythroblastosis virus E26 oncogene.
c-erv1	endogenous retroviral sequence 1.
c-ets1	avian erythroblastosis virus E26 oncogene homolog 1.
FAP	familial adenomatous polyposis coli.
c-fes	feline sarcoma (Snyder-Theilen) viral oncogene homolog.
c-fms	colony stimulating factor 1 receptor, McDonough feline sarcoma virus oncogene homolog.
c-fos	FJB murine osteosarcoma viral oncogene homolog.
c-Ha-ras	Harvey rat sarcoma viral oncogene homolog.
c-hst F1	heraran secretory transforming protein.
ICAM1	intercellular adhesion molecule 1.
c-int1	(WNT1) wingless type MMTV integration site 1, human homolog.
c-int2	murine mammary tumour virus integration site oncogene homolog.
c-jun	avian sarcoma virus 17 oncogene homolog.
c-kit	Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog.
c-Ki-ras1	Kirsten rat sarcoma 1 viral oncogene homolog.
c-Ki-ras2	Kirsten rat sarcoma 2 viral oncogene homolog.
MCC	mutated in colorectal cancer.
c-met	met proto-oncogene.
c-mos	Molony murine sarcoma viral oncogene homolog.
c-myb	avian myeloblastosis viral oncogene homolog.
c-myc	avian myelocytomatosis viral oncogene homolog.
c-N-ras	neuroblastoma RAS viral oncogene homolog.
p53	TP53 tumour protein p53.
c-raf1	murine leukaemia viral oncogene homolog 1.
c-rafB2	murine sarcoma viral oncogene homolog 2.
RARA	retinoic acid receptor alpha.
RARB	retinoic acid receptor beta.
Rb	retinoblastoma 1.
c-rel	avian reticuloendotheliosis viral oncogene homolog.
c-ros1	avian UR2 sarcoma viral oncogene homolog.
c-sis	platelet derived growth factor beta polypeptide.
c-src	avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog.
c-ski	avian sarcoma viral oncogene.
THRA1	thyroid hormone receptor alpha.
THRB	thyroid hormone receptor Beta, avian erythroblastosis leukaemia viral oncogene homolog.

Figure 6

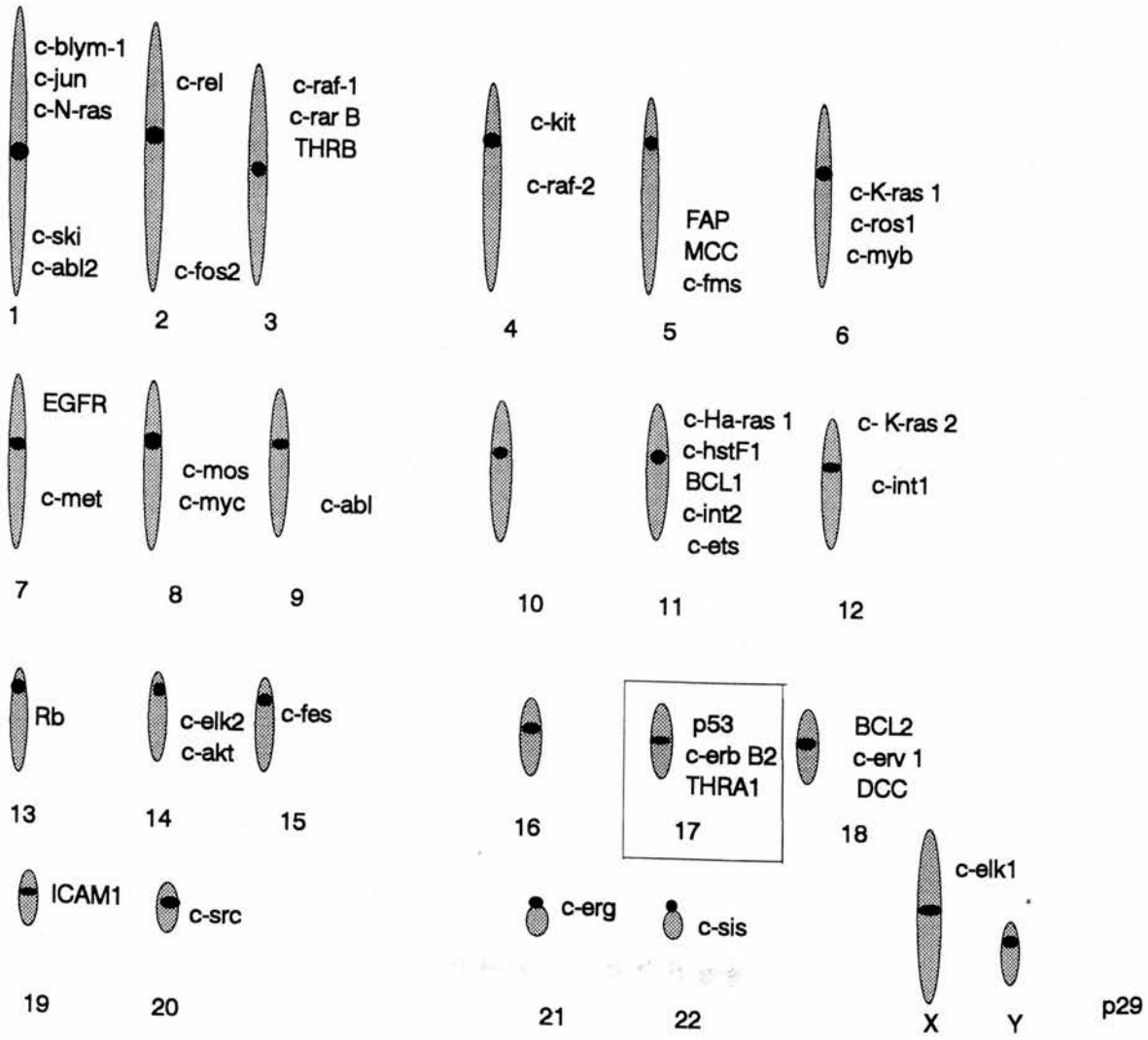
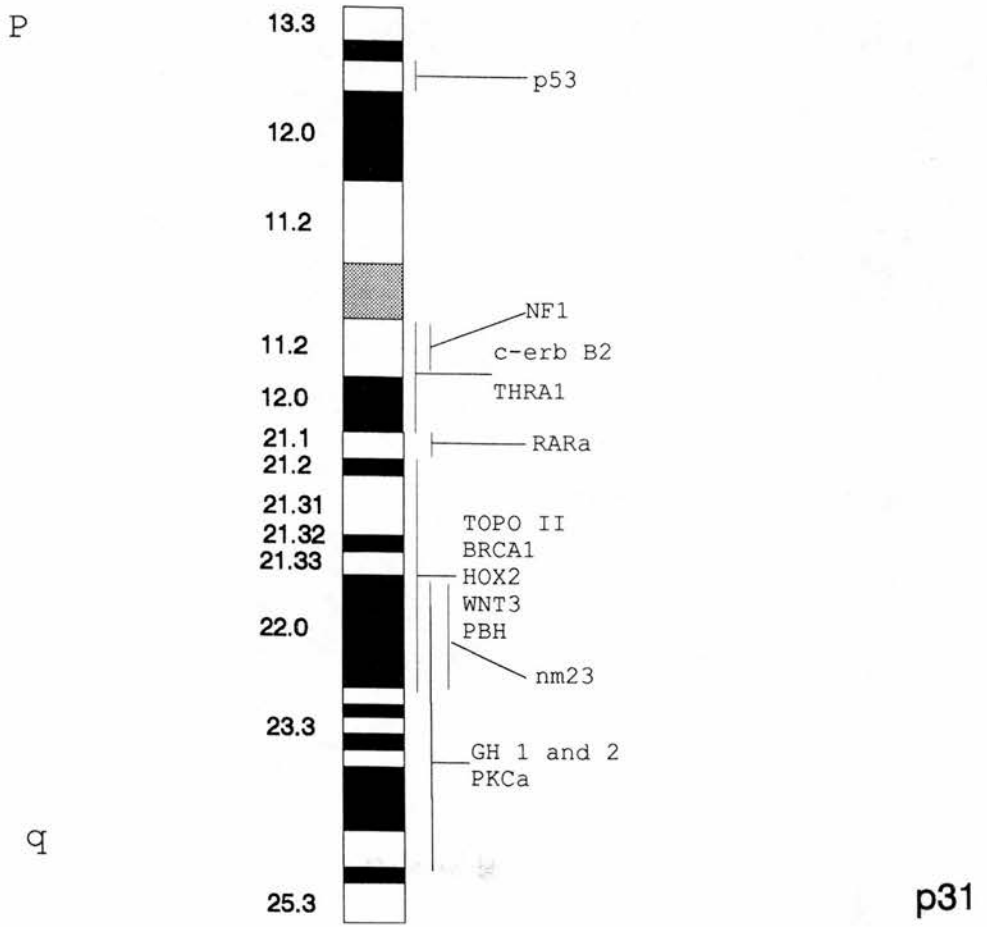

c-blym-1
c-jun
c-N-ras
c-ski
c-abl2
1
c-rel
c-fos2
2
c-raf-1
c-rar B
THRB
3
c-kit
c-raf-2
4
FAP
MCC
c-fms
5
c-K-ras 1
c-ros1
c-myb
6
EGFR
c-met
7
c-mos
c-myc
8
c-abl
9
10
c-Ha-ras 1
c-hstF1
BCL1
c-int2
c-ets
11
c- K-ras 2
c-int1
12
Rb
13
c-elk2
c-akt
14
c-fes
15
16
p53
c-erb B2
THRA1
17
BCL2
c-erv 1
DCC
18
ICAM1
19
c-src
20
c-erg
21
c-sis
22
c-elk1
X
Y
p29

Figure 7.
Oncogenes and cancer related genes located on chromosome 17. Genes illustrated are as follows;

p53	TP53 tumour protein p53.
NF1	neurofibromatosis 1.
c-erb B2	avian erythroblastic viral oncogene homolog 2.
THRA1	thyroid hormone receptor alpha
RARA	retinoic acid receptor alpha
TOPO II	topoisomerase II.
BRCA 1	breast cancer 1, early onset.
HOX 2	homeobox 2A, 2B, and 2C.
WNT3	wingless type MMTV integration site 3, human homolog.
PBH	prohibitin
nm23	non-metastatic cells 1
GH 1 and 2	growth hormone 1 and 2
PKCa	proteinkinase c-AMP dependent, catalytic alpha.

Figure 7. Chromosome 17



mice the presence of the mutated gene was shown to cause mammary carcinoma in a single step process, as demonstrated by the development of polyclonal tumours at an early age (Muller et al 1988). Other strains of mice transgenic for neu develop monoclonal tumours which metastasise, suggesting that the genome of the host animal may be influential in the development of tumours, and that additional transforming events may be required in some instances (Bouchard et al 1989).

Meanwhile, DNA cloning studies in man had identified protooncogenes called c-erb B2 and HER2. Sequencing of neu, c-erb B2 and HER2 established that they shared an identical sequence (Coussens et al 1985), and the gene was mapped to chromosome 17q21 (Fukushige et al 1986). Initial studies in human breast cancer indicated that c-erb B2 was frequently disregulated (Slamon et al 1987), however the active point mutation identified in rodents has not been found in the equivalent transmembrane coding region in human breast cancers (Lemione et al 1990). It remains possible that mutations may occur in other parts of the gene. DiFiore et al (1987) showed that c-erb B2 protein expression lacked transforming activity in NIH 3T3 cells under the control of SV40 promoter despite detectable levels of c-erb B2 protein. However a further ten fold increase in c-erb B2 protein expression in these cells under the influence of the long terminal repeat of the Molony murine leukaemia virus was associated with transformation of the cells. This was an important study as it indicated the potential for native c-erb B2 involvement in cancer development. Further studies supported the view that c-erb B2 was involved in growth promotion, with increased rates of proliferation associated with c-erb B2 overexpression in cell lines. The proliferation could be inhibited by c-erb B2 protein specific monoclonal antibodies (Chazin et al 1992).

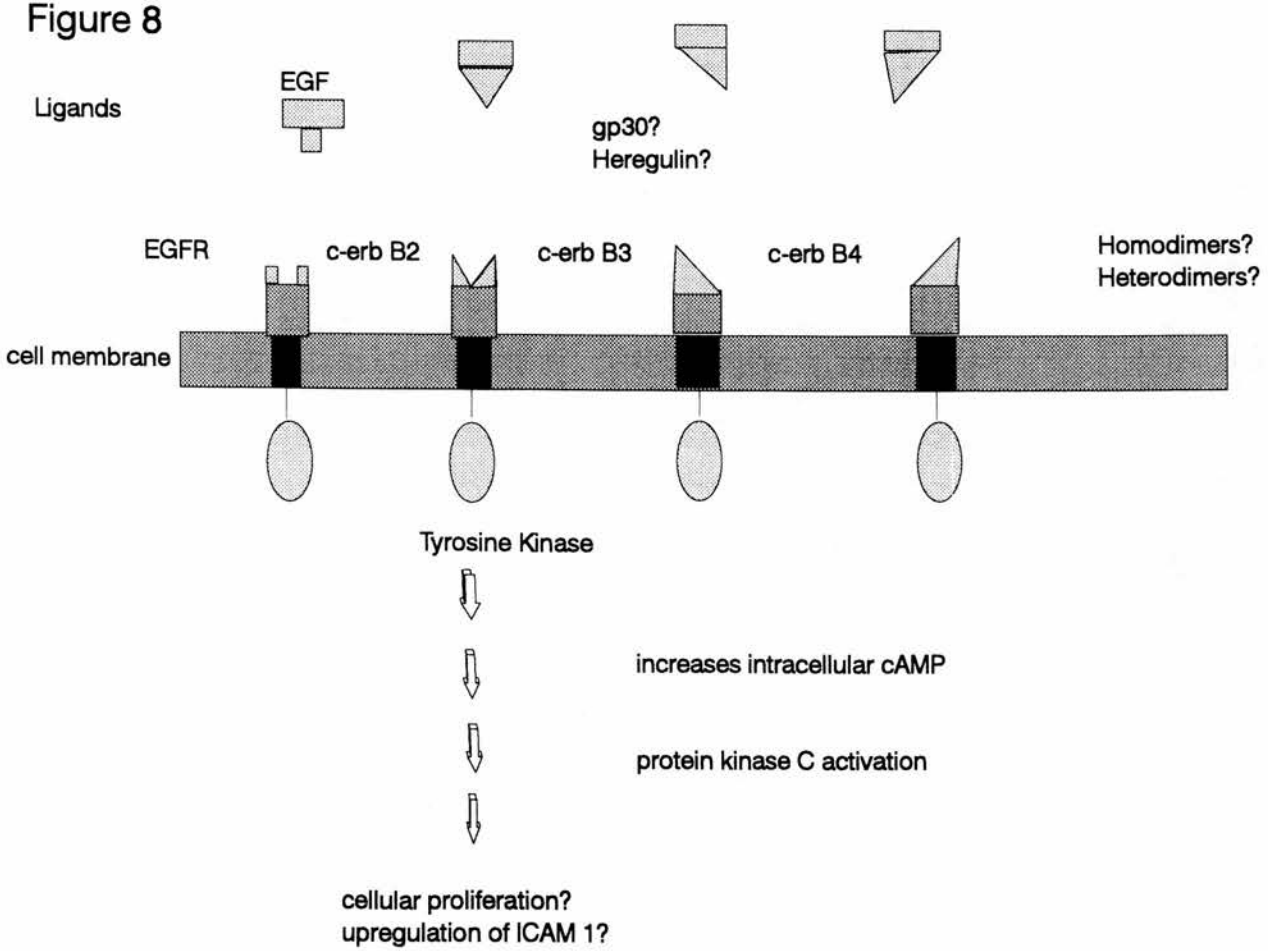
1.9.3 Biology of c-erb B2.

C-erb B2 is a protooncogene which encodes for a transmembrane receptor protein with a high degree of homology to epidermal growth factor receptor (Coussens et al 1985). The receptor is a member of the tyrosine kinase family, and which consists of an extracellular binding domain, a single transmembrane domain, a single protein tyrosine kinase domain, and at least one regulatory domain (Bargman et al 1986). Signal transduction following the binding of a growth factor to its ligand is complex, and includes internalisation of the receptor/ligand complex (Schlessinger et al 1988), followed by changes in cytoplasmic biochemistry, illustrated in Figure 8. It is not yet known if ligands bind to homodimers or heterodimers of c-erb B2 receptor. Biochemical changes include increases in intracellular cyclic AMP and degradation of phosphoinositides, which result in a release of calcium ions and the activation of protein kinase C (reviewed in Rozengurt 1986). Protein kinase C is a protein modifying enzyme which may influence the transcriptional activity of some genes, such as c-fos and c-myc (Curran and Franza 1988, Heikkla et al 1987). The c-erb B2 receptor protein is 185 kD in size and is called p185. Several ligands have been identified but the importance of the action of p185 is not understood. GP30, a 30kD growth factor secreted by MDA-231 cells (Lippman et al 1991) can stimulate phosphorylation of cells overexpressing c-erb B2 protein and can also inhibit the binding of specific antibody to c-erb B2 protein (Lupu et al 1990). GP30 can also bind and activate EGF receptor and inhibit growth of cells which overexpress EGF receptor (Hudziak et al 1989, Lupu et al 1990). Another potential ligand, heregulin, is a 45kD protein which specifically induces phosphorylation of p185. It was purified from breast tumour cell lines (Holmes et al 1992) and is similar to other proteins in the EGF family. The exact relationship between p185 , GP30 and heregulin is not clear, but it is possible that there is a family of receptors for related growth factors.

Figure 8

Ideogram showing receptor proteins of four members of the c-erb family, epidermal growth factor receptors (EGFR), c-erb B2, c-erb B3, and c-erb B4 located within the cell membrane. Receptor ligands represented include epidermal growth factor (EGF) which is specific for EGFR, and ligands gp30 and heregulin which may bind to c-erb B2. Ligands may bind to homodimers or heterodimers of c-erb receptors. C-erb B2 ligand binding to c-erb b2 receptor activate a tyrosine kinase pathway which results in an internalisation of the ligand:receptor complex, followed by changes in cytoplasmic biochemistry. These changes include increases in cyclic AMP and protein kinase C activation

Figure 8


Ligands
EGF
gp30?
Heregulin?
EGFR
c-erb B2
c-erb B3
c-erb B4
Homodimers?
Heterodimers?
cell membrane
Tyrosine Kinase
increases intracellular cAMP
protein kinase C activation
cellular proliferation?
upregulation of ICAM 1?

Specific ligands for c-erb B3 and c-erb B4 have not been identified but may include ligands for c-erb B2.

1.9.4 Existing Studies of c-erb B2 in Clinical Breast Cancer.

1.9.4.1 Introduction. Following the evidence that c-erb B2 was disregulated in rodent tumours and in a clinical series linking gene amplification with poor prognosis in breast cancer (Muller et al 1988, Slamon et al 1987), a large number of retrospective clinical studies were carried out (reviewed in Singleton and Strickler 1992). Studies of clinical samples have either measured c-erb B2 protein by specific immunohistochemistry or western blotting, assessed c-erb B2 gene amplification by Southern or dot blotting of DNA, or determined c-erb B2 mRNA concentrations in RNA extracted from samples by northern blotting. All these studies have examined the potential link between c-erb B2 disregulation and cancer prognosis. Contradictory findings have been reported for many clinical associations, and no clear picture of the involvement of c-erb in cancer prognosis has developed. Tables 1, 2 and 3 list a number of studies and their conclusions. Differences between studies may include techniques used, population of origin, type and handling of samples and variable methods of interpretations. It remains clear today that c-erb B2 is frequently considered to be disregulated in breast cancer, either by gene overexpression or gene amplification.

1.9.4.2 C-erb B2 Overexpression. C-erb B2 protein (p185) overexpression in tissues is usually assessed by immunohistochemistry using specific monoclonal or polyclonal antibodies. Recent reviews have attempted to correlate the results of the numerous studies since 1987 which assessed c-erb B2 protein expression

retrospectively in breast cancer populations (Singleton and Stickler 1992). The frequency of overexpression varied between 11 and 40%, and was more common in *in situ* cancer, with 42 to 61% of these cancers demonstrating positive staining (Barnes et al 1992, Gusterson et al 1988). The highest frequencies of overexpression have been found in the comedo subset of *in situ* cancers (77%), and in Pagets disease of the nipple (91%) (Allred et al 1992, Lammie et al 1989). Normal breast tissues do not appear to overexpress p185, even in the presence of overexpressing cancer tissue (DePotter et al 1989, Gusterson et al 1988). Other lesions of the breast, such as fibroadenoma, fibrocystic breast disease, hyperplasia with or without atypia, and metaplastic tissue have not shown p185 overexpression (Allred et al 1992, Gusterson et al 1988, Lodato et al 1990), suggesting c-erb B2 expression may act as a marker of the transition between benign and malignant disease in some breast cancers. However it remains unclear whether the contribution of c-erb B2 in this process is causal or a by-product of some other event associated with malignant transformation.

1.9.4.3 Inter-study Variability. The nature of the association between c-erb B2 overexpression and poor prognosis has been complicated by the incomparability of different studies. Poorer prognosis associated with overexpression has been found in axillary node positive cancers (Borg et al 1990, Slamon et al 1989) and alternatively in node negative cancers (Paik et al 1990, Ro et al 1989, Wright et al 1989), and is absent in other studies (Ali et al 1988b, Barnes et al 1988). Possible reasons for the discrepancies between studies and the consequent conclusions include differences in populations studied and in experimental techniques. Some of the details of the clinical sample study sets used are given in Table 1. Many studies failed to define the pathological types within their study set (Bacus et al 1990, Schroeter et al 1992, Walker et al 1989a, Wright et al 1989). Others consisted of selected groups of

cancers such as node negative (Press et al 1993), *in situ* cancers (Barnes et al 1988), or Pagets disease (Lammie et al 1989).

Differences between studies may also arise because immunohistochemistry is sensitive to differences in experimental technique, particularly to tissue fixation methods (Doyle and O'Leary 1992). Differences in antigenicity have been found in frozen and fixed breast tissue (Slamon et al 1989). The antigenicity of p185 is more effectively preserved by methacarn fixation than by formol saline (Gusterson et al 1988), however the latter is the fixative used routinely in pathology laboratories. Studies can also involve tissues from more than one laboratory, and so may include specimens which have been fixed and processed differently. Direct comparison of immunohistochemistry results may also be confounded by the subjective nature of assessing staining intensity. Criteria for scoring results range from presence or absence of stain (Penault-Llorca et al 1994), percentages of cells with positive staining (Thor et al 1989), to intensity of staining (Tsutsumi et al 1990). More recent studies have used computer aided image analysis in an attempt to quantify p185 protein (Press et al 1993).

The polyclonal antibody, 21N, is frequently used for immunohistochemical studies of c-erb B2 (Gullick et al 1987), however specific monoclonal antibodies have been developed (Mab-145ww, 3B5, 9G6, TA1, pAB1) (Beckman et al 1992, De Potter et al 1990, Konmoss et al 1990, Tsutsumi et al 1990). These antibodies, directed against different epitopes of p185, may have different binding affinities and contribute to differences in detected frequencies of overexpression between studies.

Table 1. Some studies which have assessed c-erb B2 overexpression using immunohistochemical techniques. The number of cases studied (N), antibodies used and histopathological cancer type varies between studies. Differences were detected in the frequency of c-erb B2 overexpression between Pagets disease of the nipple, insitu ductal carcinomas (DCIS), and invasive ductal carcinomas (DCI). Not all studies assessed prognostic value, but in those which did, most found an association between poor prognosis and c-erb B2 overexpression. Others found no association, or the association was restricted to cancers with or without lymph node metastasis.

Table 1

C-erb B2 Overexpression

Study	N	cancer type	c-erb B2 antibody overexpression		prognostic value
Allred et al 1992	59 649	DCIS DCI	56% 11%	21N	Yes node negative
Barnes et al 1988	72 61 148	DCIS DCI +DCIS DCI -DCIS	61% 31% 26%	21N	
Kallioniemi et al 1991	319	primary	23%	TA250, NCL-B11	Yes
Lammie et al 1989	45	Pagets	91%	21N	
Lovekin et al 1991	497	primary	15%	21N	Yes node positive
Paik et al 1990	292	primary	21%	HY83	Yes
Press et al 1993	210	DCI node negative	29%	R60, 3B5	Yes
Schroeter et al 1992	253	primary	15%	21N	Yes
Thor et al 1989	290	DCI	15%	TA1	No
Wright et al 1989	185		17%	21N	Yes node positive

1.9.4.4 C-erb B2 Expression and Cellular Proliferation. *In vitro* studies of breast cancer cell lines have shown an association between c-erb B2 overexpression and cell proliferation (DiFiore et al 1987), however the relationship between these factors in clinical cancers may not be a simple one. Comedo type *in situ* cancers have both high levels of c-erb B2 overexpression and a high rate of proliferation (Barnes et al 1988). However, attempts to correlate c-erb B2 overexpression with cellular proliferation in invasive cancers have failed to demonstrate a direct relationship (Ro et al 1989). Therefore the association of c-erb B2 activity with increased proliferation found in cultured cell lines, may not apply to all clinical cancers. C-erb B2 overexpression does not appear to be associated with changes in DNA ploidy as only weak associations with aneuploidy have been reported (O'Reilly et al 1991, Ro et al 1989)

1.9.5 C-erb B2 Gene Amplification.

1.9.5.1 Clinical Studies. Approximately 25% of invasive breast cancers contain c-erb B2 gene amplifications, although detection frequencies varied between studies from 9.1 to 33% (Clark et al 1991, Meyers et al 1990), see Table 2. Differences between study populations are similar to those discussed for c-erb B2 overexpression (section 1.9.4.3). Technical restrictions also apply to studies of gene amplification, usually measured by Southern or dot blotting. These techniques require relatively large quantities of DNA and are therefore biased towards larger cancers and low copy number amplifications may not be detectable if there is a significant contribution of normal DNA from a cancer (Ali et al 1988a). Gene amplification is generally assessed using radio labelled probes, specific for c-erb B2, which are hybridised to total sample DNA contained on a dot or Southern blot. The quantification of gene copy number is made by comparing the radiation signal intensity of the c-erb B2 test

Table 2.
Some studies which have assessed c-erb B2 gene amplification by either Southern blotting (S) or dot blotting (D) techniques. Differences between studies include number of cases studied (N), and cancer population studied. The percentages of cancers with c-erb B2 gene amplifications varied between studies, as did the prognostic value of c-erb B2 gene amplification.

C-erb B2 amplification

Study	N	cancer type	c-erb B2 amplified	method	prognostic value
Ali et al 1988	122	primary	10%	S	No
Berns et al 1992	1052	primary	19%	S	
Borg et al 1990	540		17%	S	Yes node positive
Clark et al 1991	362	primary	33%	S	Yes node positive
Meyers et al 1990	99		9.1%	S	No
Slamon et al 1987	189	primary	28%	S	Yes node positive
Tavassoli et al 1989	52	primary	28%	S D	
Ro et al 1989	66	DCI node negative	20%	S D	

gene with that of a control single copy gene, such as collagen genes (Venter et al 1987), tubulin (Corbett et al 1990), p53, or myeloperoxidase (Slamon et al 1989).

The majority of c-erb B2 gene amplifications in invasive breast cancers are small, in the order of 2 to 4 extra copies of the gene (Borg et al 1990), however, amplifications greater than 40 copies have been recorded in some invasive cancers (Ali et al 1988, Berns et al 1992, Tavassoli et al 1989). It is not known whether the degree of gene amplification or the basic amplification mechanism is significant in the biology of breast cancer, therefore an understanding of factors involved, whether active (promoting amplification) or passive (allowing amplification to occur) is important. C-erb B2 can also be amplified *in situ* cancers (Liu et al 1992) but amplification has not been detected in benign lesions (Smith et al 1994).

1.9.5.2 Gene Amplification Mechanisms. For most clinical cases the region of DNA included in an amplification unit, which includes c-erb B2, on chromosome 17 is not known. Amplifications of DNA segments including several loci on chromosome 17 have been observed in three of six invasive cancers with c-erb B2 amplification (Keith et al 1993). The amplified region included genes for topoisomerase II and retinoic acid alpha receptor and the thyroid hormone receptor gene (THRA1), all these genes have the potential to contribute to the malignant phenotype (Keith et al 1993). Retinoic acid and THRA1 are nuclear receptors whose functions include promotion of gene transcription (Bollag and Holdener 1992, Damm 1992). Topoisomerase II is an enzyme which can catalyse breakage and rejoining of DNA, and may be involved in basic cellular processes such as DNA repair, and chromosome replication (Liu 1989). This suggests that this region of DNA contains other important genes which may have a potential role in oncogenesis. That amplification of retinoic acid receptor, THRA1, and topoisomerase II have not been

observed in the absence of c- erb B2 amplification (Keith et al 1993, Tavassoli et al 1989, Tsuda et al 1989) may suggest that c-erb B2 is an essential gene within this region. This important point has not been adequately tested, and it remains possible that another critical gene is co-amplified with c-erb B2. However, the transforming activity of c- erb B2 *in vitro* (DiFiore et al 1987) and in transgenic animals (Muller et al 1988) would suggest that c-erb B2 is a relevant target. For most clinical cancers the physical nature of gene amplification is not known, although models of gene amplification, discussed above (section 1.6.4), would predict that amplifications are present as either extended chromosomal regions or extra-chromosomal elements (Stark et al 1989). *In situ* hybridisation on metaphase spreads of some breast cell cultures, incorporating c-erb B2 probes, have shown that the amplified copies of the gene are integrated at many sites in several rearranged chromosomes (Smith et al 1994), indicating that the new c-erb B2 copies may occur in abnormal locations. Whole chromosome duplication does not appear to be a major factor in c-erb B2 amplification (Paterson et al 1990, Tsuda et al 1989).

1.9.5.3 C-erb B2 Messenger RNA. The presence of elevated levels of p185 on cancer cell membranes could be the result of different disregulatory mechanisms, such as posttranslational protein folding, the presence or absence of ligand, or increased gene transcription. The concentration of c-erb B2 mRNA within clinical samples of breast cancer is estimated by reaction of extracted RNA in Northern blots using radio labelled c-erb B2 cDNA probes. Also, excess c-erb B2 mRNA can be detected within tissue sections by reactions of these probes by *in situ* hybridisation. Excess c-erb B2 mRNA has been detected in 30 to 43% of invasive breast cancers (May et al 1990, Parkes et al 1990), and in 61% of invasive breast cancers with lymphocytic infiltration (inflammatory cancers) (Guerin et al 1989).

Studies which concurrently assessed c-erb B2 gene amplification and mRNA found that gene amplification could only be clearly detected in approximately half those cancers where excess mRNA was apparent (Guerin et al 1989, King et al 1989, Parkes et al 1990). This suggests that transcriptional control mechanisms and not gene amplification may be responsible for excess mRNA in a proportion of breast cancers. In addition, mRNA production is not always in proportion to the c-erb B2 copy number (King et al 1989) indicating that abnormal transcriptional control mechanisms may also be active in the presence of gene amplification.

1.9.5.4 Combined Studies of c-erb B2 Gene Amplification and Overexpression. Table 3 lists those studies which matched the results of c-erb B2 gene amplification and p185 overexpression in individual invasive breast cancers. In three large studies, approximately eighteen percent of breast cancers showed both amplification and overexpression (Borg et al 1990, Ciocca et al 1992, Slamon et al 1989). However, recent studies detected greater frequencies of overexpression (up to 50%) and amplification (up to 38%) (Iglehart et al 1990, Kerns et al 1990). These high frequencies may reflect technical improvements, such as the use of more sensitive antibodies.

These results suggest that the majority of c-erb B2 gene amplifications may lead to functional expression of the protein. However, the data also suggest that a proportion of cancers which overexpress p185 may not show gene amplification (Borg et al 1990, Iglehart et al 1990, Kerns et al 1990), and conversely, amplification can occur without overexpression (Ciocca et al 1992, Uehara et al 1990). This suggests that for some cancers, mechanisms which turn on or off genes are likely to play an important role in the effect of c-erb B2 in breast cancer. The direct

Table 3. Some studies which have compared c-erb B2 gene overexpression and c-erb B2 gene amplification. N= cases studied

C-erb B2 overexpression and amplification

Study	N	cancer type	c-erb B2 expression	c-erb B2 amplification
Borg et al 1990	300	primary	19%	17%
Ciocca et al 1992	1103	primary	17%	19%
Corbett et al 1992	79	primary	25%	31%
Yamada et al 1989	50	primary	38%	20%
Venter et al 1987	43		26%	33%

relationship between c-erb B2 expression and c-erb B2 gene amplification remains unclear, due to the inconsistent findings between studies. This thesis aims to clarify this relationship with the development of better methods of detecting gene amplification, see below.

1.9.6 Differential PCR.

A sensitive determination of gene amplification in an unbiased breast cancer population is necessary for an informed analysis of the role of c-erb B2 in breast cancer. Improved sensitivity may be possible by adapting the polymerase chain reaction (PCR) for the detection of gene amplification in fixed breast cancer tissues. A major advantage of this technique is that all cancers, including those too small for analysis by Southern blotting, can be tested for gene amplification.

The polymerase chain reaction (Saiki et al 1988) has facilitated detailed study of specific genes, and has been further developed to detect gene amplifications (Frye et al 1989). The standard polymerase chain reaction cannot be regarded as quantitative due to the exponential nature of the reaction and high sensitivity to minor changes in reaction conditions (Saiki et al 1988). Differential PCR was developed to semiquantify gene copy numbers in breast cancer (Frye et al 1989), using cell lines with a known amplification of several genes, including c-erb B2 and the epidermal growth factor receptor. The technique involves co-amplification of a single copy reference gene with the target gene within one reaction tube. An estimation of gene copy is indicated by the ratio between the quantities of the two PCR products. The differential PCR developed by Neubauer et al (1992) used a complex algorithm of four different differential PCR reactions for detection of c-erb B2 amplification in fixed breast tissue. Using this method, Liu et al (1992) detected amplification of c-erb B2 in 25 of 122 node positive invasive cancers (21%), and 13 of 27 *in situ*

cancers (48%). Using a one step differential PCR, c-erb B2 amplification was detected in 40% of ovarian cancers (Hruza et al 1993), compared to the 3 to 21% previously found by Southern blotting analyses (Imayanitov et al 1992, Zhang et al 1989). This indicates that dPCR can be a very sensitive method of detecting gene amplification in cancers, in a wide range of clinical samples.

1.9.7 C-erb B2 and Cancer Progression.

The role of c-erb B2 in breast cancer initiation and progression remains unknown. Inconsistencies between studies and lack of information on its biological functions, make its contribution to cancer biology difficult to assess and there remains confusion over its prognostic significance. A model of cancer progression involving c-erb B2 has been proposed by Allred et al (1992). They hypothesised that c-erb B2 plays an important role in initiation of breast cancer, and that overexpression decreases within individual tumours as they evolve from *in situ* to invasive lesions; alternatively he suggests that many invasive carcinomas arise de novo, without an *in situ* stage, and not involving disregulation of c-erb B2.

The identification of "early" and "late" events in cancer progression has been difficult due to the considerable heterogeneity of breast cancer. However, histopathological features such as type, grade, lymph node status and size, along with biochemical features such as oestrogen receptor status (see section 1.11) may form a chronological framework. The relationship between these features and c-erb B2 disregulation will be addressed in this thesis, with a view to assessing the role of disregulated c-erb B2 in breast cancer progression.

1.10 Nuclear Receptors.

1.10.1 Introduction.

The contribution of c-erb B2 to stages of breast cancer progression may be due to molecular mechanisms which switch on and off gene expression. Nuclear receptors, in particular oestrogen receptors, may be directly or indirectly involved in the regulation of c-erb B2 expression. Another nuclear receptor, the thyroid hormone receptor can be co-amplified with c-erb B2.

Genes and their products which influence the activation or suppression of other genes are known to be important in the development of the malignant cell phenotype. One major family of such genes, known as *trans* acting genes, are the nuclear receptor genes, including the genes for steroid receptors, thyroid hormone receptor (THRA1, also known as c-erb A) and vitamin D receptors (King 1992).

1.10.2 Nuclear Receptor Biology.

The products of nuclear receptor genes generally operate within the nucleus of the cell (reviewed in King 1992). When complexed with their ligand, they can directly influence the transcription of target genes. Nuclear receptor genes all encode proteins of a similar basic structure, with a carboxyl terminal region which binds ligand, a transactivation region and a DNA binding region which binds the target gene DNA. The active portion of the latter region consists of an amino acid sequence capable of binding zinc ions in two loops, known as zinc fingers. Nuclear receptors become active upon binding their specific ligand. The receptors then shed their associated heat shock proteins (hsp) allowing the receptor to perform DNA binding. The receptors can bind to target DNA via recognition sites within the zinc finger, but the method of alteration of transcriptional control is presently unknown. It is possible

that the transactivation region becomes capable of stabilising general transcription factors, such as TFIID, following DNA binding (O'Malley et al 1990). DNA binding is also associated with receptor dimerization and tetrameric structures are also known to occur (Tsai-Pflugfelder et al 1988).

1.10.3 Nuclear receptor zinc fingers.

Zinc fingers consist of a region of up to 30 amino acids formed into two loops which co-ordinate a zinc ion within the centre of each loop. These amino acid regions are highly conserved between receptors and are critical for DNA binding specificity (Mader et al 1989), positive control of transcription (Schena et al 1989) and DNA dependant dimerisation (Umesono et al 1989). Small groups of amino acids within these loops have specific functions such as binding to the phosphate backbone of DNA and binding to specific DNA bases. It is suggested that alterations in zinc finger sequences may play a part in altered receptor function in dysfunctional cells (Freedman and Luisi 1993). For example, mutations in the DNA binding region of the vitamin D receptor are associated with reduction of receptor activity and consequent hereditary rickets (Hughes et al 1991). Similarly, mutations in the DNA binding region of the androgen receptor are present in patients with testosterone insensitivity syndrome (Trapman and Brinkman 1993). It is therefore possible that other mutations in the DNA binding region of other nuclear receptors, such as THRA1 or oestrogen receptor, may decrease or increase the consequent action of the target gene, or may alter the specificity of the DNA binding region for its target gene site.

1.10.4 Response Elements.

Nuclear receptors bind to specific cis-acting DNA sequences, called response elements, usually located within the promoter region of target genes (Damm 1992).

Table 4.

Consensus DNA binding sequences for glucocorticoid, oestrogen, and thyroid hormone receptor.

Hormone Receptor	Consensus DNA Binding Sequence
Glucocorticoid	**AGAACAnnnTGTTCT**
	TCTTGTnnnACAAGA
Oestrogen	**AGGTCAnnnTGACCT**
	TCCAGTnnnACTGGA
Thyroid	**AGGTCATGACCT**
	TCCAGTACTGGA

The response elements that react with different nuclear receptors all have a similar DNA structure, consisting of two hexameric half-sites separated by three DNA bases. Although the sequences of the half-sites vary considerably in nature, consensus sequences for some receptors have been determined (King 1992) and are shown in Table 4. The consensus thyroid receptor response element is clearly identical to the oestrogen receptor element, minus three intervening bases (Glass et al 1988).

Although these consensus sequences are not fixed (some consensus sequences include only one n base between the thyroid receptor element half-sites) there is clearly a great deal of similarity between the response elements to which nuclear receptors bind (O'Malley et al 1990). The palindromic nature of the response elements indicate that receptors are bound as dimers. Alteration in the number of intervening bases between the half-sites of the response element can inhibit dimerisation of the respective receptor (Freedman and Luisi 1991), probably due to altered spatial interactions between the two molecules.

Regardless of the similarity of their response elements, receptors remain able to discriminate between different target genes, even when response elements differ by only two base pairs, as do glucocorticoid and oestrogen receptor response elements. The ability of each receptor to specifically discriminate is determined by the identity of three amino acids in the DNA binding region of the first zinc finger (Danielson et al 1991, Mader et al 1989, Umesono et al 1989). It is also possible that response elements are not the only mechanism for interaction between receptors and DNA sequences. The production of mRNA of the oncogene c-myc can be up-regulated by the presence of oestrogen and consequent oestrogen receptor activity in cancer cells (Dubik et al 1992). However, analysis of the c-myc promoter region in these cells,

did not locate a response element corresponding to known receptor response elements, see above. It is possible therefore, that in addition to the mechanisms outlined above nuclear receptors can act on oncogene DNA sequences with no homology to recognised response elements.

1.11 Oestrogen receptors.

Oestrogen has a major hormonal role in the normal and cancerous breast (Anderson 1989, Hulka et al 1994). The association of oestrogen hormones with cancer risk indicate that hormonal status can be important in the aetiology of breast cancer (Hulka et al 1994) as discussed previously (section 1.3.4). Oestrogen receptors are typical nuclear receptors and are present in 6 to 10 % of normal breast epithelial cells, but in reduced concentrations after menopause (Petersen et al 1987). Many invasive cancers have detectable levels of oestrogen receptor, and such cancers are generally thought to have a better prognosis (Habel and Stanford 1993). However, approximately 40 percent of invasive breast cancers have no detectable oestrogen receptors (Habel and Stanford 1993). Anti-oestrogen therapy, such as tamoxifen, is currently used to treat oestrogen receptor positive breast cancers, and a good response to treatment is anticipated (Early Breast Trialists Collaborative Group 1992). Tamoxifen competitively binds to oestrogen receptors, reducing the cell growth response associated with the action of oestrogen on breast cancer cells. Tamoxifen binding does not inhibit receptor binding to its response element, but does inhibit its transactivating functions (Fuqua 1994).

The heterogeneity of oestrogen receptor concentration and differing responses to hormonal treatment suggests that there may be many ways in which oestrogen and 1994). Indeed oestrogen and oestrogen receptor are involved in a complex pathway,

which includes the conversion of oestrone to oestradiol, the binding of oestrogen to oestrogen receptor in the cell nucleus, and the binding of this complex to oestrogen response elements in target genes, resulting in a block or enhancement of gene oestrogen receptor can be disregulated in breast cancer (Fuqua 1994, Hulka et al transcription (Hulka et al 1994).

It is possible that oestrogen receptors are lost during carcinogenesis (Malet et al 1989), however the loss of oestrogen receptors is not thought to be due to deletion of the relevant gene (Piva et al 1988). The loss may be due to reduced transcription of the gene following its methylation or mutation, or to defective transcriptional mechanisms. Oestrogen receptor variants have been reported in a small number of breast cancers (Murphy and Dotzlaw 1989) and may be due to post-translational mechanisms such as alternative splicing (Miksicek et al 1993). Mutations have been found within the receptor coding region of the gene in clinical breast cancers (Fuqua et al 1991), indicating that some cancers may have non-functional oestrogen receptors.

Target genes for oestrogen receptor action may also be important, however few specific target genes have been identified for oestrogen receptor. Studies in cultured cells have identified transforming growth factor alpha, transforming growth factor beta, insulin like growth factor 1, and somatomedin C as potential target genes (Barrett-Lee 1991), however there are likely to be many more. The up-regulation of gene transcription by an oestrogen-oestrogen receptor complex may increase cellular proliferation (Barrett-Lee 1991) therefore genes which have proliferative functions may be potential targets. A positive association between c-erb B2 overexpression and absence of oestrogen receptor has been observed in some breast cancer studies

(Borg et al 1990, Tandon et al 1989), although other studies have found a less clear cut correlation (May et al 1990, Gullick et al 1991, Slamon et al 1987, Zeillinger et al 1989). The relationship between oestrogen receptor status and both c-erb B2 gene amplification and overexpression is addressed in this thesis.

1.12 THRA1.

Another nuclear receptor, the thyroid hormone receptor gene alpha (THRA1), has been associated with disregulation of c-erb B2, however this association is a physical association involving co-amplification of both genes in some breast cancers (Tavassoli et al 1989). The THRA1 gene (C-erb A1) codes for the thyroid hormone receptor, and is located closely distal to c-erb B2 on chromosome 17 (Laudet et al 1991), both being assigned to the same band, 17q21. The thyroid receptor has a typical nuclear receptor structure and mode of action.

The oncogenic potential of THRA1 was first established in its viral counterpart v-erb A, a mutant form of the THRA1 gene which alters the neoplastic phenotype of erythroid cells transfected with the avian erythroblastosis virus (Glass et al 1988, Graf and Beug 1983). v-erb A enhances the activity of v-erb B in transformation of avian erythroid cells, but can not transform cells without the presence of v-erb B (Damm, 1987).

Breast cancers can have amplification units containing both c-erb B2 and THRA1, however a functional relationship between these genes has not been demonstrated in human breast cancer. Attempts to measure THRA1 mRNA in c-erb B2-THRA1 amplified cancers have failed to show any increase (Tavassoli et al 1989), suggesting that gene transcription does not occur even in the presence of increased gene copy

number. In addition, THRA1 is always amplified with c-erb B2 and is never present as a single gene amplification unit (Tavassoli et al 1989, Tsuda et al 1989). This may suggest that amplification of THRA1 is coincidental and that selection pressure may be relevant to only to c-erb B2 or another local gene. However it remains possible that mutations may exist in the THRA1 gene in breast cancer. This thesis investigates potential co-amplification of THRA1 with c-erb B2, and examines the zinc finger coding exons of THRA1 for functional mutations.

1.13 Other Factors

As previously discussed, nuclear receptors and oncogenes may be involved at a fundamental level of the oncogenic process, switching on or off target genes which may contribute to the malignant phenotype. In addition, there are many other biological indicators of cancer progression. Diverse enzymes are produced by malignant cells and are implicated in cancer cell invasion and include: collagenases (Stetler-Stevenson 1990), stromelysins (McDonnel and Matrisian 1990); cathepsin B (Sloane et al 1990); cathepsin D (Rochefort et al 1990); heparinase (Nakajima et al 1987); and urokinase plasminogen activator (Testa and Quigley 1990). These enzymes, usually proteases, may play a role in metastatic dissemination of cancer cells by contributing to basement membrane and connective tissue degradation. Other important genes are those which code for transcriptional regulators of proteases, such as the tissue inhibitor of metalloproteases (TIMP) (McDonnel and Matrisian 1990), oestrogen receptor (Hendry et al 1990), PAI 1, PAI 2 and protease nexin (Hart and Rehmtulla 1988). A recently discovered serpin, called maspin, may also be a protease inhibitor (Zou et al 1994). Maspin is produced in mammary epithelial cells, but is absent in breast cancer cells, involved lymph nodes and distant

metastasis, suggesting that it may have tumour suppressing activities (Zou et al 1994).

Cathepsin D, an acidic lysosomal protease, is produced and secreted by breast cancer cells. High concentrations of cathepsin D have been associated with lymph node metastasis and suggested as a marker of poor prognosis (Brouillet 1990). However, expression of cathepsin D protease is inducable by oestrogen, may be associated with prolonged survival in oestrogen receptor positive breast cancers (Henry et al 1990). Stromelysin 1, 2 and 3 represent a family of related metalloprotease genes, they can be overexpressed in breast cancer cells (McDonnel and Matrisian 1990). Expression of stromelysins may not be restricted to cancer cells, indeed stromelysin 3 can be expressed in fibroblastic cells of tumour stroma immediately surrounding cancer cells (Basset et al 1994).

Amongst such genes is that coding for urokinase plasminogen activator (uPA) which is frequently overexpressed and associated with aggressive behaviour in breast cancer cells (Duffy et al 1988, Janicke et al 1990). Disregulation of uPA, whether directly by physical alteration to the gene structure or indirectly, via transcriptional mechanisms may play an important role in the metastasis of the malignant cell.

1.13.1 Urokinase Plasminogen Activator.

Urokinase Plasminogen activator (uPA) is one of a family of plasminogen activators which includes tissue type plasminogen activator (tPA). Both are involved in the breakdown of the extracellular matrix and may play a role in the promotion of cellular dissemination (Sumiyoshi et al 1991). However, analysis of tPA and uPA concentrations in breast cancer has indicated that it is uPA rather than tPA which is

involved in lymphatic dissemination (Yamashita et al 1993). Several large studies have observed an association between the presence of uPA and aggressive behaviour in breast cancer (Foekens et al 1992, Grondahl-Hansen et al 1993, Janicke et al 1990). The mode of action of uPA has been intensively studied and several transcriptional control mechanisms have been identified (Saksela and Rifkin 1988).

1.13.2 Biology.

uPA is a serine protease enzyme present in the cytoplasm of epithelial cells. A single high molecular weight chain forms a proenzyme precursor, which is converted to an active two chain uPA (chain A and B) by plasmin cleavage of a peptide bond (Schmitt et al 1992). Conversion to the two chain form may take place after the binding of the single chain form to its cell surface receptor, which is a highly glycosylated protein anchored to the plasma membrane (Schmitt et al 1992). This bound complex is more active than free uPA in degradation of the extracellular matrix (Hearing et al 1988, Ossowski 1988, Schlechte et al 1989).

Single chain, double chain, and receptor bound uPA are all active forms of the enzyme, and can convert plasminogen to plasmin, which can degrade proteins of extracellular tumour stroma (Saksela and Rifkin 1988). uPA can also activate procollagenase which in turn degrades collagen and basement membrane proteins (Murphy et al 1992). These abilities suggest that uPA could be involved in the dissemination of cells and contribute to the formation of metastases in cancer progression.

1.13.3 Genetics.

The gene for uPA is 6,387 nucleotides long and consists of eleven exons separated by ten introns (Riccio et al 1985). uPA has been localised to the distal third of the long arm of chromosome 10 (q24-qter)(Tripputi et al 1985). Several promoter or enhancer regions have been identified in the 5' flanking sequence of the gene (Cassady et al 1991, Riccio et al 1985). Multiple weak cis-acting (promoter or enhancer binding) elements are distributed over a large portion (7kb) of the 5' flanking sequence (Cassady et al 1991). This indicates that transcription of uPA may be under the influence of many promoter and enhancer elements. Post translational mechanisms may also regulate the concentration and activity of uPA in breast cancer cells, perhaps by increased stability of mRNA (Henderson et al 1992).

1.13.4 Clinical Significance.

Initial indications that uPA may be involved in the carcinogenic process came from experiments on human ovarian cancer cells, which released uPA into the culture medium (Astedt et al 1976). Elevated uPA has been reported in affected tissues in colon (Corasanti et al 1980), lung (Markus et al 1980), prostate (Camiolo et al 1981) and breast cancers (Duffy et al 1988, Evers et al 1982, Foekens et al 1992, Janicke et al 1990). In breast cancer the rise in plasminogen activator is solely due to a rise in uPA and is not associated with the tissue form of plasminogen activator (Layer et al 1987). Immunohistochemical and biochemical studies on breast cancers have shown that overexpression of uPA occurs in cancer cells but not in the surrounding stroma (Duffy et al 1988, Foucre et al 1991, Janicke et al 1990), or in normal cells, except for macrophages (Pyke et al 1993). uPA immunohistochemical staining is generally localised to the cell membrane and the cytoplasm and is usually present diffusely throughout affected malignancies(Janicke et al 1990). Metastatic cells appear to

have a similar concentration of uPA to those in the primary lesion (Janicke et al 1990).

Different frequencies of overexpression and different quantities of uPA have been observed in breast cancer studies. Expression of uPA has been detected in 28% to 100% of breast cancers using immunohistochemical techniques (Clavel et al 1986, Janicke et al 1990) for elevated uPA. uPA concentrations measured by ELISA in cancer cell cytosols have also shown increased uPA concentrations, varying from four to nineteen times greater than normal breast tissue (Duffy et al 1990, Foucre et al 1991, Janicke et al 1990, Sumiyoshi et al 1991). Consequently studies which examined possible associations between elevated uPA and clinical markers of cancer aggression in breast cancer have found variable or no correlations. Positive correlations with cancer size and number of axillary nodes with metastasis were found in one study (Duffy et al 1990) but not in others (Foucre et al 1991, Janicke et al 1990).

The biological consequences of elevated uPA may therefore depend on other factors such as the presence of specific inhibitors of uPA within cancer cells and the concentrations of uPA receptors on the cell surface (Yamashita et al 1993). uPA activity can be controlled by the inhibitors PAI-1, PAI-2, PAI-3 and protease nexin (reviewed in Hart and Rehemtulla 1988), and some breast cancers show highly increased levels of PAI 1 and 2 (Foucre et al 1991). Maximum uPA activity may also require the presence of uPA specific cell surface receptors (Ossowski et al 1992) which can be present in increased concentrations in invasive cancers (Del Veccio et al 1993, Pyke et al 1993). The inhibitory and enhancing affects on uPA by other factors, means that no clear conclusion about the controlling influences on uPA in breast cancer is possible. Disregulation may occur in the uPA gene, as well as the

many other factors that can promote or inhibit its action. Gene amplification, as a possible mechanism for uPA disregulation, is examined in this thesis using differential PCR (see section 1.9.6), and related to uPA protein expression measured immunohistochemically. The potential effects on uPA expression by oestrogen receptor status and c-erb B2 expression will be explored.

1.14 Aims.

Many important questions in breast cancer progression remain unanswered, particularly those pertaining to the genetic events which give rise to different pathologies, and different prognostic outcomes. Models of cancer development could include one or more initiating events, with diverging or converging pathways of genetic consequences which may share common disregulatory events. Difficulties arise in identifying early through to late stage developments in breast cancer. The early stages of cancer development may be better detected in a screened population, where cancers may be detected before they become symptomatic. Accordingly this thesis addresses the following unresolved problems:

1. Critical determination of c-erb B2 gene amplification, using differential PCR, and overexpression (Chapters 2 and 3).

2. Investigate the relationship between c-erb B2 gene amplification and clinical and pathological features of breast cancers (Chapter 4).

3. Investigate the relationship between oestrogen receptors and c-erb B2 amplification and overexpression (Chapter 4).

4. Examine possible allelic imbalance of c-erb B2 in c-erb B2 amplified tissues (Chapter 5).

5. Examine the THRA1 gene for possible mutations of functional significance (Chapter 6).

6. Investigate disregulation of the urokinase plasminogen activator gene (Chapter 7).

CHAPTER 2

Validation of Differential PCR for the Determination of Gene Amplification.

2.1 INTRODUCTION

Since the publication of a study linking c-erb B2 to poor prognosis in breast cancer patients (Slamon et al 1987), many studies have examined c-erb B2 gene amplification, as measured by Southern or dot blotting (reviewed in Perren 1991; Singleton and Strickler 1992). However there are major differences between studies in the association of c-erb B2 disregulation with histopathological features and with prognosis, therefore its involvement in cancer development and progression is difficult to determine. It is not clear whether differences in results between studies have been due to variations in sample selection, experimental technique or to genuine disparity between populations of biological relevance. It is therefore important to establish robust and accurate techniques with which to measure gene disregulation.

Of the techniques for measuring gene amplification, Southern and dot blotting suffer from the disadvantage of requiring microgram quantities of DNA for analysis. Tissue morphology is also destroyed in the extraction process. The development of polymerase chain reaction (PCR) technology has made possible the molecular analysis of minute quantities of DNA (Saiki et al 1988). The PCR reaction consists of repeated cycles of heating to over 90^{o}C denaturing target DNA to single strands, annealing (hybridisation) of specific oligoprimer DNA segments to the target gene and extension of the remaining DNA segment by thermoactive DNA polymerase, resulting in precise duplication of the target sequence, illustrated in Figure 9. However, accurate quantitative measurement of gene copy number is compromised by the sensitive and exponential nature of the PCR reaction. The concentration of PCR amplified DNA is defined by the equation $x= I(1+E)n$, where x is the concentration of PCR amplified DNA, I is the concentration of target DNA prior to PCR, E is the average efficiency for each cycle, and n is the number of thermal

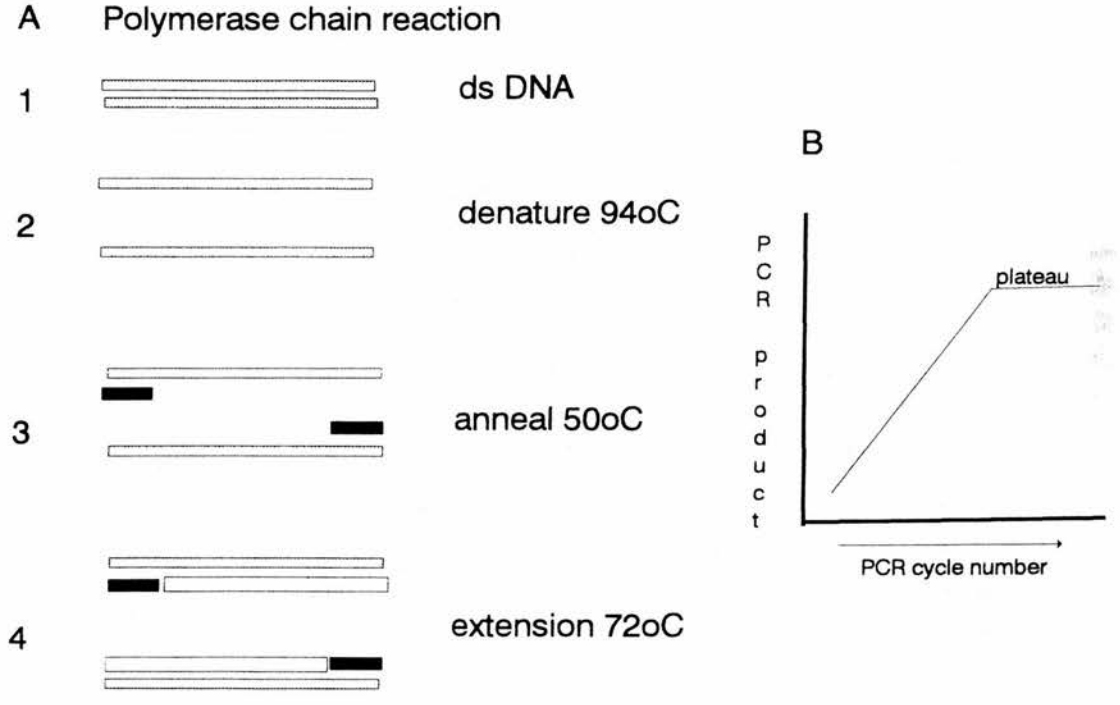
amplification cycles. The efficiency of the reaction can theoretically range from 0 to 1, and can be affected by parameters such as the temperature of annealing, concentration of primers or template DNA, the formation of secondary DNA structures, primer-primer bonding or inhibition. The quantity of PCR products can also vary between duplicate DNA samples using the same concentrations of reagents and identical cycling parameters (Gilliland et al 1990), due to minor differences in template/reaction mixture concentrations or minor differences in thermal profiles between samples. The efficiency of primers binding to the DNA template appears to be a critical factor in determining the final quantity of PCR product (Sugimoto et al 1993). To overcome this clear drawback of PCR for measurement of gene amplification, a semi-quantitative estimation of gene copy number using a differential PCR for DNA in cell lines was developed (Frye et al 1989). Differential PCR introduces an internal control into each reaction tube i.e. a known single copy gene is co-amplified with the test gene. The ratio between the intensity of detectable bands produced from each gene is calculated, which can give an estimation of the test gene copy number. Differential PCR has been extended to the analysis of DNA extracted from fixed, paraffin processed breast cancer tissue (Liu et al 1992, Neubauer et al 1992) however, this technique is not yet fully characterised for clinical samples, indicating that validation of dPCR is required for each new gene application.

Optimal template DNA preparation is important for PCR analysis. Previous methods of preparing formalin fixed tissue for PCR have included various procedures such as dewaxing, proteinase K digestion and phenol/chloroform extraction (Goelz et al 1985, Jackson et al 1990, Shibata et al 1992, Wright and Manos 1990). Methods employing proteinase digestion of tissues are time consuming (e.g. 5 day incubations, Jackson et al 1990) and those employing freeze-thaw cycles (Wright and Manos 1990) may partially destroy valuable DNA. Breast cancer tissues in this study were

Figure 9

An ideogram of the polymerase chain reaction, illustrating specific sequence amplification. Double stranded template DNA (1) is denatured at 94oC (2), specific primers anneal to template DNA at 50^{o}C (3), and Taq polymerase extends the DNA sequence at 72^{o}C, resulting in a duplication of the original sequence. Products increase at an exponential rate throughout the thermal cycle course (B) until one or more reaction reagents become limiting, effectively stropping the reaction (plateau phase).

Figure 9


A
Polymerase chain reaction
1
ds DNA
2
denature 94oC
3
anneal 50oC
4
extension 72oC
B
PCR product
plateau
PCR cycle number

fixed in methacarn, a fixative known to preserve DNA (Doyle and O'Leary 1992, Jackson et al 1990). However, DNA preparation of methacarn fixed tissue for PCR has not been fully explored.

This chapter explores the sensitivity of differential PCR in detecting an increased c-erb B2 gene copy number in cell lines. Differential PCR is a relatively new technique and factors, such as primer efficiency, reagent concentrations, and DNA template concentration, are unknown variables within the reaction. The application of dPCR to fixed, paraffin embedded tissue requires an investigation of optimal DNA preparation methods. Possible inaccuracies introduced from fixed tissue, such as degraded DNA or possible alteration of the gene copy number of the reference gene also need to be investigated.

2.2 MATERIALS AND METHODS

2.2.1 DNA Samples.

2.2.1.1 Test Samples. Breast cancer tissue was obtained fresh at the time of operation from samples removed for biopsy or mastectomy. Excess fat was trimmed and selected portions of each cancer were snap frozen in liquid nitrogen and stored at -70°C; adjacent portions were fixed in methacarn (6:3:1 methanol:chloroform: acetic acid) overnight at 4°C for subsequent processing in alcohol and xylene by standard methods and embedded in paraffin blocks. Samples used for optimisation of experimental protocols varied between experiments due to the limited quantity of tissue available for study.

2.2.1.2 Control DNA. Control DNA derived from normal human placenta (p258) was a kind gift of Dr. E. Thompson, MRC Human Genetics Unit, Edinburgh. Normal human female DNA derived from placental chorionic membrane, was also obtained commercially (Sigma, UK). Both Sigma and p258 control DNAs would be expected to have a single copy of c-erb B2. Throughout this thesis the term single copy is used to mean one gene per haploid genome. It is understood that normal cells will therefore possess 2 c-erb B2 genes, on the homologous pair of chromosome 17. Control tissues were also obtained from fresh breast tissue distant to the lesion site or from non-cancer bearing breasts. These were fixed in methacarn and processed as above.

2.2.1.3 Cell Lines. Human breast cancer cell lines known to have c-erb B2 amplification were used to calibrate the relationship between differential PCR ratio

values and gene copy number. The epithelial cell line 21MT2 was obtained from Dr R. Sager, Dana-Farber Cancer Institute, Boston, MA. and contains a 40 fold amplification of the c-erb B2 gene (Band et al 1989). The cell line UISO BCA1 was obtained from Dr R.R Mehta, the University of Illinois, Chicago, IL. and contains a 10 fold increase in the c- erb B2 gene (Sasi et al 1991). MCF7, a cell line derived from breast carcinoma epithelial cells was obtained from Gibco, U.K. MCF7 is reported to be hemizygous (Frye et al 1989), i.e. has a "half gene dosage" of c-erb B2 in the nomenclature adopted here.

Each cell line was cultured from cryo-preserved cell stocks at 37°C in air with 5% CO_2 added. 21 MT2 was cultured in alpha MEM (Gibco) containing 10% foetal calf serum, 2mM L-glutamine, 1mM sodium pyruvate, 0.1 mM non essential amino acids, 1 μg/ml insulin, 2.8 μM hydrocortisone, 12.5ng/ml epidermal growth factor and 10mM HEPES. UISO BCA1 and MCF7 were cultured in GMEM (Gibco), 10% foetal calf serum and 2mM L-glutamine, with supplementary oestrogen (10ng/ml, Sigma, U.K.) for MCF7 only.

2.2.2 DNA Preparation and Concentration.

2.2.2.1 Frozen Tissue and cell lines. For DNA preparation from frozen breast tissue samples, approximately one gram of tissue was removed, chopped and pulverised while frozen, then immersed in 0.1ml of a digestion buffer, Tris-SDS-proteinase K, pH 7.5 (see Appendix 2) and incubated for 18h at 37°C. DNA was extracted by standard phenol/chloroform extraction (Sambrook et al, 1989), precipitated on addition of 0.1 volume of 5M sodium acetate, and 2 volumes of cold absolute ethanol (see Appendix 2), washed twice in 70% ethanol and dried under

vacuum. DNA was reconstituted in 0.01M Tris-EDTA buffer, pH7.6 (see Appendix 2) to a final concentration of 1mg/ml.

Cell lines MCF7, UISO BCA1 and 21MT2 were harvested from semi-confluent monolayer cultures in exponential growth phase by removal of media and addition of 5% Trypsin in phosphate-buffered saline (Gibco,U.K.). After trypsinisation for 5 minutes at 37^{o}C, cells were removed and washed twice in Tris buffered saline (see Appendix 1). DNA was extracted from the washed cells using digestion buffer as described above.

All the above DNA concentrations were measured in a Beckman spectrophotometer, at 260nm extinction in quartz cuvettes.

2.2.2.2 Paraffin embedded tissues. Methods of DNA extraction from paraffin embedded breast tissue have not been previously standardised for use in quantitative assessment of gene dosage, therefore three different methods were investigated. They are fully described in section 2.3.1 of results and are based on: 1. boiling in water, 2. lysis in Tris-EDTA-Tween buffer containing proteinase K, and 3. boiling in lysis buffer. For each breast cancer tissue tested four 10μm tissue sections from each specimen were cut on a Reichert-Jung 2030 microtome and separately transferred to sterile eppendorf tubes. Frozen sections were cut on a Bright cryostat (Huntingdon, UK), and transferred to sterile eppendorf tubes on ice. Care was taken to clean the microtome blade with xylene between samples.

2.2.3 Differential PCR.

The reaction dynamics of dPCR with primers for c-erb B2 and interferon gamma 150 (IFNG150, single copy reference gene) were tested experimentally. Optimisation of a number of factors was required before undertaking a large clinical study using this technique. These included sample preparation (section 2.3.4), PCR amplification conditions, primer efficiency, dPCR assessment of template DNA degradation, and investigation of reference gene integrity.

2.2.3.1 Primers and the Polymerase Chain Reaction. Primers used in the differential PCR were short segments of DNA whose sequences were specific for either IFNG150, interferon gamma 82bp (IFNG82), interferon beta (IFNB), or c-erb B2. Nucleotide sequences and chromosomal origin are given in Table 5. The single copy reference gene selected for the standard dPCR was the interferon gamma 150bp gene.

2.2.3.2 Standard Differential PCR Reaction. The standard differential PCR was performed on a Techne PHC3 thermal cycler. Each reaction vial contained five µl of prepared DNA template suspension, derived from the tissue sections as described in section 2.8, or from 200ng of DNA extracted from frozen tissue (except where stated otherwise), 0.25nM of the two primers for each gene (total of 1nM), 0.2M of mixed nucleotide bases, dNTPs (Pharmacia, U.K.), 3µCi 32P dCTP, x1 *Taq* polymerase buffer and one unit of *Taq* polymerase (Northumbria Biotechnology Ltd, U.K.). The total volume in each PCR reaction tube was 50µl. Cycling parameters were one cycle of 94°C for 5 mins, 50°C for 1 min, 70°C for 1min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, 70°C for 1 min and one cycle of 94°C for 1min , 50°C for 1 min, 70 °C for 5 mins.

Table 5.

The origins and DNA sequences of PCR primers for genes c-erb B2, Interferon gamma (IFNG150= 150 base pair amplicon), Interferon gamma (IFNG82= 82 base pair sequence), and Interferon beta (IFNB).

Gene	Primer sequence	Chromosome	Reference
c-erb B2			
Sense	5'- CCT CTG ACG TCC ATC ATC TC-3'	17q21	Frye et al
Antisense	5'- ATC TTC TGC TGC CGT CGC TT-3'		(1989)
IFN-γ150			
Sense	5'- TCT TTT CTT TCC CGA TAG GT-3'	12q24.1	Frye et al
Antisense	5'- CTG GGA TGC TCT TCG ACC TC-3'		(1989)
IFN-γ82			
Sense	5'- GTG TCT CCT CCA AAT TGC TC-3'	12q24.1	Neubauer
Antisense	5'- GCC ACA GGA GCT TCT GAC AC-3'		et al(1992)
IFN-β			
Sense	5'- GCA GAG CCA AAT TGT CTC CT-3'	9p22	Neubauer
Antisense	5'- GGT CTC CAC ACT CTT TTG GA-3'		et al(1992)

To ascertain the relative quantities of the test gene to the reference gene, a standardised method of determining the quantity of each PCR product was required. In all experiments, PCR products were separated by size, in duplicate, on 2% agarose gels (3:1 Nuseive/Seakem, FMC, UK) at 125V for 2 hours, stained with ethidium bromide, and photographed. Where dPCR reactions included a radiolabel, ^{32}P dCTP, visible bands were excised, finely chopped and transferred to separate scintillation vials, to which 5mls of Optiphase-safe scintillation fluid was added. A value for the quantity of PCR product was derived from the radioactivity present in each vial, assessed as counts per minute (CPM) on a Beckman scintillation counter. CPM values were calculated in duplicate for these test samples in repeat experiments, to assess intra and inter experimental variation.

2.2.3.3 Measurement of PCR Product. Two methods of measuring PCR products, UV densitometry and radio-isotope incorporation, were compared. Standard dPCR was performed incorporating 1μCi ^{32}P, using primers for c-erb B2 and IFNG150, on DNA suspensions derived from 16 methacarn fixed breast cancer tissues, DNA extracted from frozen tissue from sample CR214, and the control DNA p258.

2.2.3.4 Calculation of Ratio Values. Results from dPCR were expressed as ratio values. For example, for the UV densitometry method, these were calculated by dividing the c-erb B2 PCR product value by the IFGN150 reference gene value. For the radio incorporation method, ratio values were calculated by dividing the corrected average CPM for c-erb B2 by the average CPM for IFNG150. Corrected CPM values for each PCR product were obtained from the duplicate gel tracks after subtracting the average experimental blank (PCR reaction with no template DNA) and applying a correction factor of 1.25 to compensate for differences in dCTP

content between IFNG150 (69 C bases) and c-erb B2 (55 C bases). Similar correction factors were calculated and applied to dPCR involving other combinations of primer pairs, as shown in Table 6.

Table 6.
Correction factors applied to PCR products for the determination of dPCR ratio values. This was necessary in order to make equivalent the number of ^{32}P labelled dCTP bases within each amplicon. The correction factor was calculated by dividing the number of CTP bases in amplicons created by primer set B by the number of CTP bases in the amplicon created by primer set A. The correction factor was applied to CPM derived from PCR product from primer set B. Primer sets represented are interferon gamma 150bp = IFNG150, interferon gamma 82bp = IFNG82, interferon beta = IFNB, c-erb B2 oncogene = c-erb B2, and urokinase plasminogen activator (see Chapter 7) = uro.

primer set(A)	C1	primer set(B)	C2	c2/c1 correction factor
IFNG150	**69**	**c-erbB2**	**55**	**1.25**
IFNG150	**69**	**IFNG82**	**30**	**2.3**
IFNG150	**69**	**IFNB**	**54**	**1.27**
IFNG150	**69**	**uro**	**66**	**1.04**

2.3 RESULTS.

2.3.1 DNA Preparation from Paraffin Sections.

To establish an optimal method for preparing DNA for the PCR from methacarn-fixed tissue, three methods were assessed and applied to four 10μm sections from each of 5 breast cancer specimens. Methacarn fixed and frozen fresh tissue sections were tested from each cancer. Five microlitre aliquots of each of these preparative methods was added as DNA template to a standard PCR reaction (section 2.2.3.2) using primers for c-erb B2 and interferon gamma 150. PCR products were electrophoresed at 125V for 2h on 2% agarose gels, stained in ethidium bromide and photographed.

Method 1: Tissue sections were boiled in 0.1ml of sterile water for 10 minutes at 100°C in a boiling water bath. PCR product was not detectable for either fixed or frozen tissues.

Method 2: Tissue sections were dewaxed in two extractions with one ml of xylene, washed twice in 70% ethanol, dried under vacuum, then incubated overnight at 37°C in 0.2ml of a Tris-EDTA-Tween lysis buffer, pH8.5 with added proteinase K (see Appendix 1), then boiled for 8min in a boiling water bath. Supernatant fluid contained sufficient template DNA to allow successful PCR amplification from both fixed and fresh template. However, these supernatant fluids were unstable when stored at -20°C, and some failed to produce PCR products when thawed and retested.

Method 3: Tissue sections were boiled in 0.1ml of lysis buffer (50mM Tris pH8.4, 1mM EDTA, 0.5% Tween 20) for 8 minutes at 100°C in a boiling water bath. Supernatent fluids, from both fixed and fresh tissue, contained template DNA which was readily PCR amplified, and produced consistent PCR results. Differential PCR ratio values were unaffected by multiple freeze thaw cycles (x10), permitting several PCR reactions from each specimen preparation. Therefore, this method was used for all subsequent DNA preparation from tissue sections.

Further testing of specimens prepared by method 3 examined the optimal number of paraffin sections in each 0.1 ml preparation (1, 2, 3 and 4 10μm sections of breast cancer CR254), and the volume of supernatant containing template DNA required in dPCR (5, 10, 20μl per dPCR reaction). A standard dPCR reaction was performed using primers for c-erb B2 and IFNG150, and ratio values calculated (as in section 2.2.3.2). Differential PCR ratio values obtained for 1, 2, 3, and 4 μm sections were 3.1, 2.5, 2.7, and 2.8 respectively. Five, 10 and 20μl of template DNA produced ratio values of 3.1, 4.4, and 4 respectively. This indicated that the number of sections and the volume of supernatant fluid included in each dPCR reaction did not greatly affect the ratio values obtained by standard dPCR. For all subsequent tests, four sections per fixed tissue sample were boiled for 8min in lysis buffer and 5μl of supernatant fluid used in each dPCR.

2.3.2 Comparison of Template DNA Derived from Fixed and Frozen Tissue in dPCR.

The possible effects of fixation on dPCR ratio values was investigated. Four 10μm sections of four methacarn-fixed breast cancer tissues, cases CR46, CR53, CR90 and

CR214, were prepared in lysis buffer (method 3 above). Two hundred ng of DNA was extracted from a frozen sample of the same tissues and suspended in 5µl of the 0.01M Tris-EDTA buffer. Five µl of each suspension was added to separate vials and a standard dPCR performed (section 2.2.3.2). Differential PCR ratio values from frozen and methacarn fixed tissue from the same case varied only slightly (Table 7), indicating that tissue fixation does not distort these ratio values. The high ratio value derived from frozen DNA in case CR90 was probably due to degraded template DNA (see below).

2.3.3 Development of Differential PCR and Investigation of Reaction Efficiencies.

2.3.3.1 Measurement of dPCR Products.

Two methods of measuring dPCR products, UV densitometry and radio-incorporation, were compared. Standard dPCR was performed on 16 methacarn fixed breast tissues, 1 DNA from frozen tissue, and one DNA control (p258), incorporating 1µCi ^{32}P, and primers for c-erb B2 and IFNG150. Products were separated on agarose gels (section 2.2.3.2).

2.3.3.1.1 UV Densitometry. For ultraviolet densitometry, each gel was scanned by video camera, and the images digitised and stored in a computer file (software supplied by UVP Products, UK). All subsequent calculations were made from this stored image. The program automatically deleted a computed background fluorescence for each gel. A value for the intensity (quantity) of each PCR product was derived from the peak height and peak area either manually or automatically.

Table 7

Differential PCR ratio values indicating c-erb B2 gene amplification status in template DNA derived from frozen or fixed breast tissue specimens.

Specimen	dPCR ratio value	
	DNA (fresh)	Fixed (methacarn)
CR46	**3.0**	**2.6**
CR53	**1.5**	**1.1**
CR90	**9.4**	**3.8**
CR214	**3.8**	**3.1**

UV densitometry readily measured strongly stained bands, however tracks containing faint bands became indistinguishable from the background values, Figure 10. Ratio values derived from densitometry peaks are given in Table 8. For faint bands, manual evaluation of peak heights was possible but delimitation of areas under the curve was subjective, see Figure 10. Also, uneven illumination from the UV transilluminator occurred, making photographs of the same gel, positioned on different parts of the transilluminator result in different densitometry peaks, see Table 8.

2.3.3.1.2 Radio-incorporation. One, 3 or 5 μCi of ^{32}P dCTP was added to separate PCR reactions containing template DNA from breast cancer case CR157, and standard differential PCR performed as previously described. PCR products were electrophoresed and visible bands were excised and CPM counted as described in section 2.2.3.2. Duplicate tracks were assessed for intraexperimental variation.

The optimal ^{32}P dCTP concentration was considered to be 3μCi to minimise radiation whilst maintaining acceptable label incorporation into nucleotides used in the dPCR. There was little variability of the ratio values determined by radio incorporation in duplicate gel tracks, see Table 9.

2.3.3.1.3 Comparison of UV Densitometry and Radio-incorporation. For the comparison of the two techniques, the differential PCR was performed on DNA from 14 breast cancers and one control DNA (p258), incorporating 1μCi ^{32}P radio labelled dCTP in the standard PCR reaction mixture, section 2.2.3.2. The PCR products were separated on agarose gels, then stained and photographed for digital recording for UV densitometry analysis, before bands were excised for scintillation counting as described above. Ratio values obtained by each method for the same

Figure 10

Gel photographs and computer overlay of ultraviolet densitometry analysis of PCR products. The photographs show analysis of two different gel tracks from the same agarose gel, one which contains densely stained PCR product (A), and one which contains faintly stained PCR product (B). The lower photographs represent analysis of the complete gel track, the upper photographs a computer enhanced signal from the same track. Excessive "background noise" is illustrated in photographs B, where PCR product bands are faint.

Figure 10

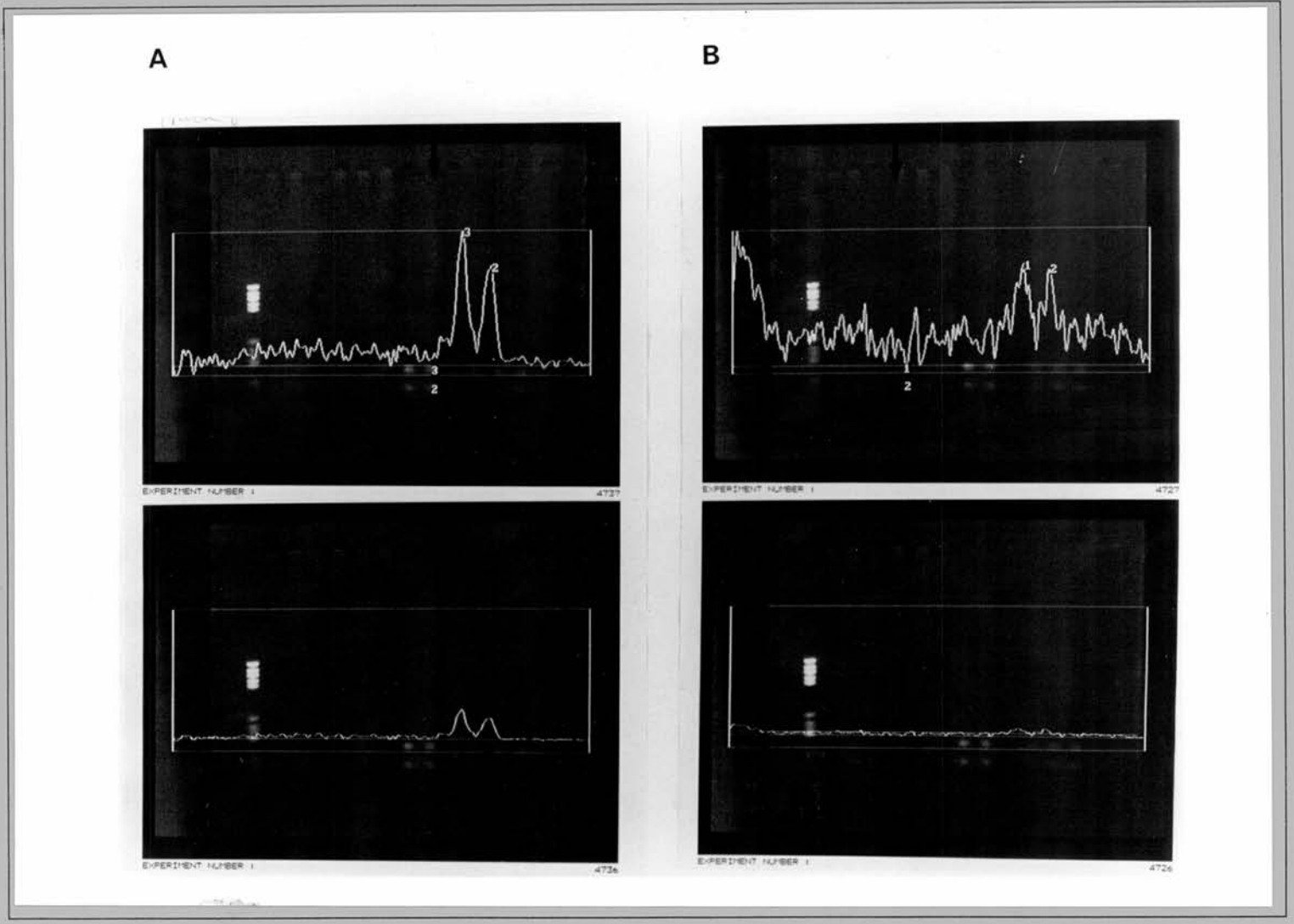

A
B
3
2
3
2
2
EXPERIMENT NUMBER 1
4727
EXPERIMENT NUMBER 1
4727
EXPERIMENT NUMBER 1
4726
EXPERIMENT NUMBER 1
4726

Table 8.
Comparison of three methods of determining the ratio values of the PCR products of cerb B2/IFNG150 in 14 fixed and one frozen breast cancer tissues and a control DNA (p258). Ultraviolet ratio values were calculated from either the peak height (UV peak) or the area under the peak curve (UV area) of PCR products detected by densitometry. The values in parentheses are those from a second densitometric analysis of the same gel in a different position on the trans illuminator. Ratio values determined from radiolabel incorporation (^{32}P) were calculated from corrected CPM detected by scintillation.

Table 8

Specimen	ratio UV peak	ratio UV area	ratio 32P
CR173	**2.6**	**3.1**	**2.4**
CR174	**2.7**	**3.3**	**2.6**
CR175	**1.3**	**NR**	**1.1**
CR176	**2.3**	**2.6**	**2**
CR179	**5**	**NR**	**3.1**
CR180	**1.2**	**1.6**	**1.2**
CR197	**2.7**	**3.24**	**1.7**
CR199	**3.3**	**4.06**	**1.6**
CR202	**0.6**	**0.64**	**1**
CR204	**3.9 (2.2)**	**5.78 (2.37)**	**2.8**
CR205	**NR(1.5)**	**NR (1.57)**	**1.4**
CR212	**2.3**	**2.47**	**2.8**
CR213	**2.1**	**2.68**	**2.5**
CR214	**3.8**	**5.8**	**5.9**
CR214dna	**4**	**5**	**5.2**
p258	**1.4**	**1.37**	**1.2**

NR = No result determined.

Table 9

Differential PCR ratio values for c-erb B2 gene copy number in 6 breast cancer specimens demonstrating consistency in ratio value calculation from duplicate gel tracks.

Specimen	dPCR ratio value Track 1	Track 2
CR174	**2.03**	**2.15**
CR176	**2.05**	**2.28**
CR197	**2.32**	**2.23**
CR199	**2.55**	**2.68**
CR202	**1.08**	**1.23**
CR205	**1.42**	**1.48**

dPCR reaction were compared.

The comparison of ratio values obtained by ultra violet densitometry and by the radio-incorporation method showed a good correlation, see Table 8. Dissimilar results were only observed in the PCR products of two samples, CR197 and CR199. Analysis of PCR products by UV densitometry was also extremely time consuming and was not used further. Radio incorporation was therefore the method of choice for quantitation of PCR products and subsequent determination of ratio values because of its accuracy and consistency.

2.3.3.2 Optimal Concentration of Template DNA in dPCR.

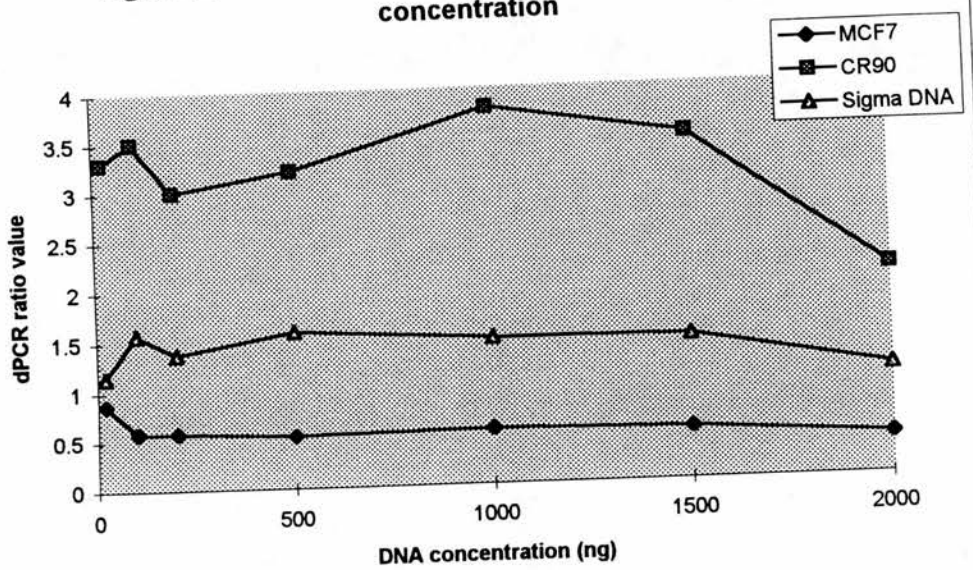
A number of experimental variables were assessed in order to identify factors which might influence dPCR ratio values. The efficiency and perturbation of the dPCR reaction with respect to template DNA concentration, was tested in the standard dPCR incorporating primers for IFNG150 and c-erb B2, in separate vials containing 25, 50, 100, 200, 500, 1,500 or 2,000 ng of control DNA p258, cell line DNA MCF7, and breast cancer DNA CR90. Ratio values for each test DNA concentration were calculated as described in section 2.2.3.3.

All concentrations of template DNA tested gave detectable dPCR products from primers c-erb B2 and IFNG150, but at 25ng, bands were noticeably weaker. The tolerance of the dPCR for high concentrations of template DNA (100ng to 2,000ng) is shown in Figure 11. Ratio values were similar over the range of 100 to 1500 ng of template DNA. Sample CR90, which produced a high ratio value in a previous experiment, maintained its high value throughout this range, but showed a reduced, but still amplified ratio value for 2,000ng. This may indicate that c-erb B2 primers

Figure 11

Titration of template DNA concentration in differential PCR reactions incorporating primers for c-erb B2 and IFNG150. Fifty, 100, 200, 500, 1000, 1500 and 2000ng of template DNA of cell line MCF7 (hemizygous for c-erb B2), control Sigma DNA (one copy c-erb B2), and breast cancer cr90 (amplified c-erb B2) were tested in a standard dPCR reaction. Differential PCR ratio values did not vary greatly with DNA template concentrations between 50 and 1500ng DNA.

Figure 11 Effect on dPCR ratio values of DNA template concentration



are exhausted over the last thermal cycles and IFNG150 primers continue to produce product with each cycle, thereby reducing the final ratio value. These results indicate that the PCR primers were not rate limiting, except when template DNA was >1,500ng.

Attempts to estimate the DNA concentration in the fluid released from paraffin sections, by spectrophotometry, proved unrewarding.

2.3.3.3 Tolerance of dPCR to Different Concentrations of *Taq* Polymerase and dNTPs.

The effect on dPCR ratio values of different concentrations of nucleotides or *Taq* polymerase was assessed. 0.25, 0.5, 0.75, 1.0, 2 and 3 units of *Taq* polymerase were added to separate reactions incorporating 200ng DNA from control DNA (Sigma) and cell line UISO BCA1. Ten, 20, 50, 100, 200, and 500 mM of mixed dNTPs were added to separate reactions incorporating 200ng control DNA (Sigma). Ratio values for the quantity of gene copies measured for each parameter were calculate as described in section 2.2.3.3..

Variation in the initial concentrations of *Taq* polymerase or dNTPs added to the dPCR reaction indicated that ratio values were not greatly affected for concentrations between 0.25 and 1 units *Taq* polymerase and between 20 and 400mM dNTPs, see Figures 12 and 13 respectively. High concentrations of *Taq* polymerase may have had an inhibitory effect on PCR amplification, indicated by reduced total PCR product, possibly due to increased concentration of glycerol which is present in the enzyme storage buffer. These experiments indicate that minor variations in the concentration of these reaction elements are not likely to affect dPCR ratio values.

Figure 12

Titration of Taq polymerase concentration in a standard dPCR incorporating primers for c-erb B2 and IFNG 150, for cell line UISO BCA1 and control Sigma DNA.

Figure 13

Titration of dNTP concentration in a standard dPCR, incorporating primers for c-erb B2 and IFMG150, with cell line DNA UISO BCA1 and control Sigma DNA.

Figure 12 Titration of Taq polymerase in dPCR

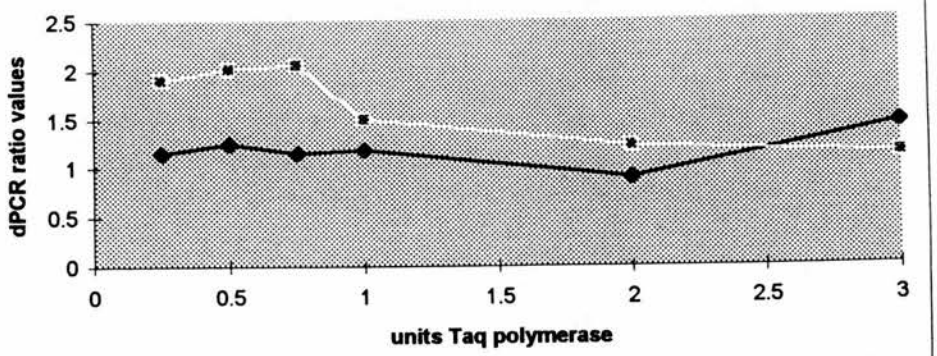
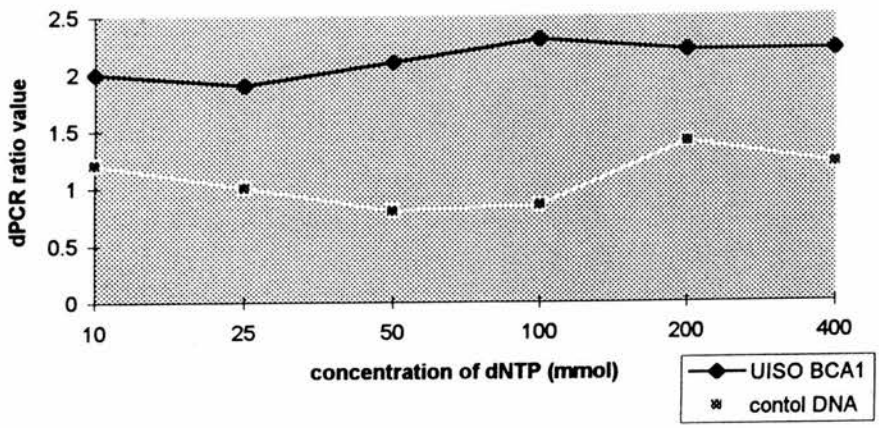


Figure 13 Titration of dNTP concentration in dPCR



2.3.3.4 Primer Efficiency of c-erb B2 and IFNG150 in dPCR

The relative efficiencies of the two sets of primers in dPCR reactions were assessed by titration of the c-erb B2 primers and by monitoring the two relevant PCR products throughout the progression of PCR cycles.

125, 250 and 500 pmol of each c-erb B2 primer with 250 pmol of IFNG150 primer were used in separate PCR reactions containing 200ng control DNA (Sigma) or cell line DNA UISO BCA1 and ratio values for each primer concentration calculated as described in section 2.2.3.2. Optimal concentrations of the primer pairs to provide a ratio value of approximately one for control DNA were considered to be 250pmol, see Figure 14.

For cycle monitoring, the total volume in each PCR reaction tube was increased to 0.2ml, with primer concentrations of 250 pmol each, and other reagent concentrations kept as above. Two hundred nanogram of template DNA prepared from frozen samples of breast cancer cases cr189 or cr342 was used and the standard dPCR performed as described in section 2.4.2. Samples (20μl) were removed from PCR reaction tubes on completion of 20, 23, 25, 27, 29, 31, and 35 cycles. Ratio values were determined for primer pair c-erb B2 and IFNG150 as described below. Assessment of dPCR product throughout a cycle course showed that ratio values remained similar for both DNAs tested, Figure 15.

These experiments indicate that primers for c-erb B2 and IFNG150 work at similar efficiencies in dPCR.

Figure 14

Titration of c-erb B2 primers (125, 250, and 500 pmol) in a standard dPCR reaction containing 250pmol IFNG150 primer, with cell line DNA UISO BCA1 and control DNA (Sigma).

Figure 15

Differential PCR ratio values obtained throughout a thermal cycle course for breast cancer samples cr342 and cr189 in a standard dPCR reaction. PCR product was sampled at 2 cycle intervals between cycles 20 and 36.

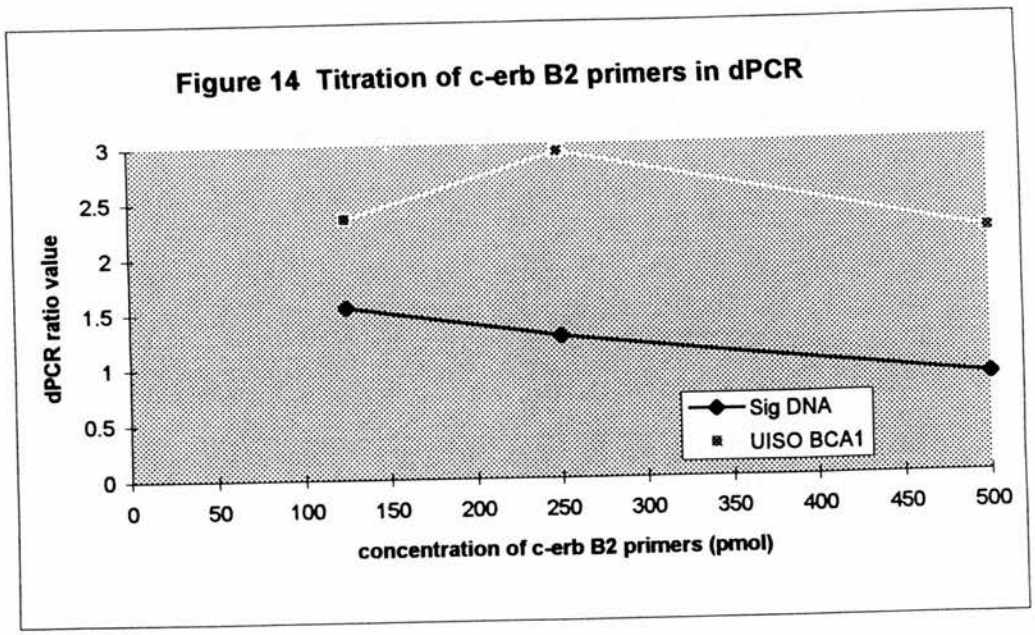


Figure 14 Titration of c-erb B2 primers in dPCR
dPCR ratio value
3
2.5
2
1.5
1
0.5
0
0
50
100
150
200
250
300
350
400
450
500
concentration of c-erb B2 primers (pmol)
Sig DNA
UISO BCA1

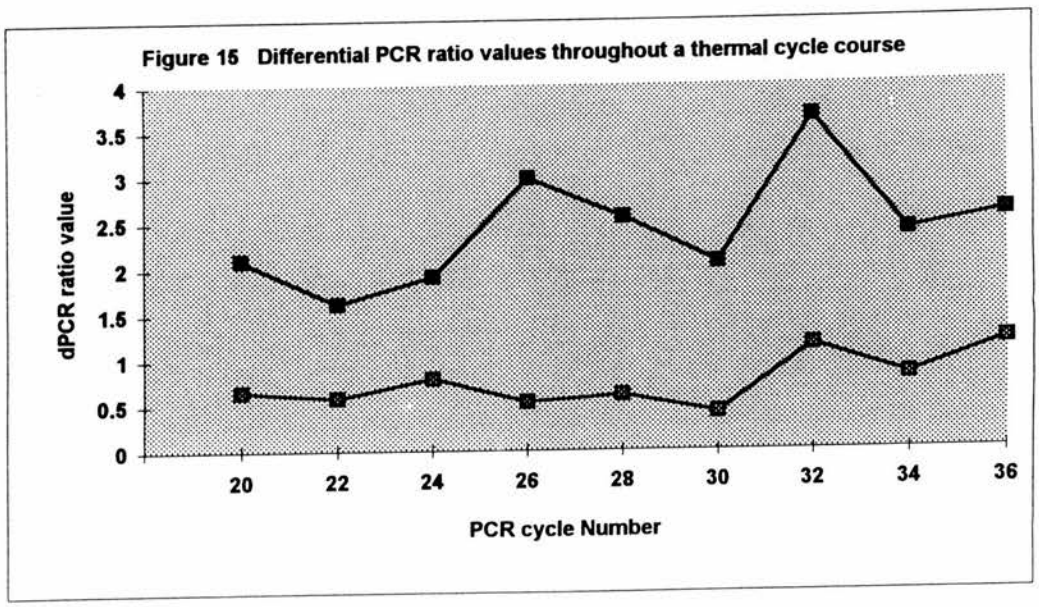


Figure 15 Differential PCR ratio values throughout a thermal cycle course
dPCR ratio value
4
3.5
3
2.5
2
1.5
1
0.5
0
20
22
24
26
28
30
32
34
36
PCR cycle Number
cr342
cr189

2.3.4 Calibration of Differential PCR Ratio Values.

Differential PCR calibration experiments were performed on DNA derived from both fixed and fresh cell culture preparations in order to determine the relationship between gene copy number and dPCR ratio value, and to further test the effect of fixation on dPCR ratio values.

2.3.4.1 Cell Line DNA

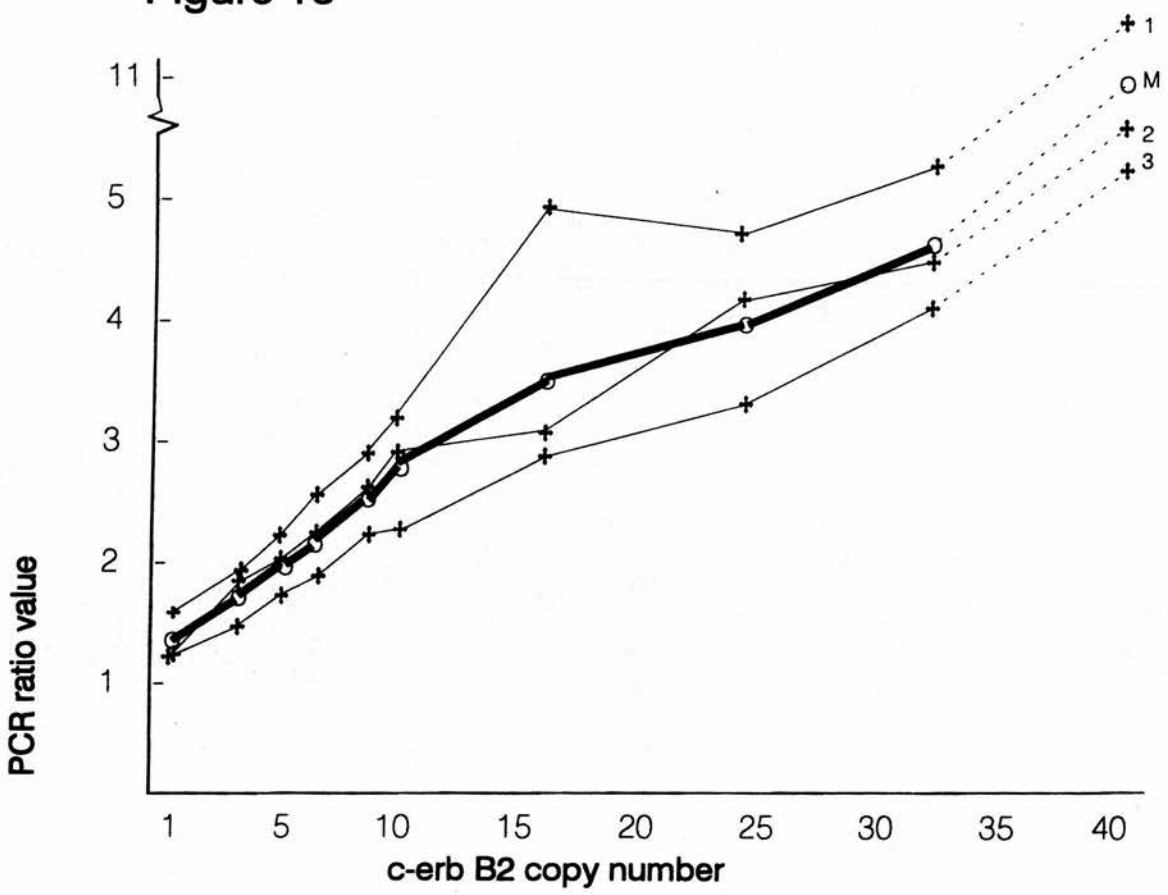
For calibration of ratio value with gene copy number, DNA was prepared from cell lines as described in section 2.2.1.3. DNA extracted from cell line 21MT2 or cell line UISO BCA1 was mixed with control DNA p258, one c-erb B2 gene copy, in separate tubes, in proportions which gave a series of known c-erb B2 copy numbers. The 21MT2 DNA was diluted to produce vials containing c-erb B2 copy numbers of 32, 24, 16, and eight. UISO BCA1 was diluted to give copy numbers of 9, 6, 5, 4, and three. The standard differential PCR was performed with primers for c-erb B2 and IFNG150, and ratio values determined by radio incorporation as described in section 2.2.3.2..

The c-erb-B2/IFNG150 ratio values derived from the above DNA solutions of known c-erb-B2 gene copy number are shown in Figure 16. As defined here, "one gene copy" corresponds to the normal diploid content of one cell. Increasing gene copy number resulted in increasing ratio values, values for 1 and 40 gene copy numbers were 1.66 and 11.46 respectively. A ratio value of 2 apparently corresponded to approximately 5 copies of the c-erb B2 gene. Therefore each ratio value is a derived value that does not equate with, but is proportional to the copy number.

Figure 16

Relationship between c-erb B2 copy number and differential PCR ratio value. Each copy was derived by dilution of DNA from c-erb B2 amplified cell lines 21MT2 or UISO BCA1 with placental DNA (one copy). Each point on line M (bold) represents the mean of triplicate experiments. Individual experiments are represented by lines 1, 2, and 3.

Figure 16


PCR ratio value
11
5
4
3
2
1
1
5
10
15
20
25
30
35
40
c-erb B2 copy number
1
M
2
3

2.3.4.2 Fixed cell line DNA.

To ascertain if fixation could produce variation in ratio values, pellets of cultured cells from the tested cell lines were fixed in methacarn. Measured suspensions of 21 MT2 cells were mixed with measured suspensions of MCF7 cells to give projected c-erb B2 copy numbers of 6, 4, 2 or 0.5 (MCF7 alone); a control DNA (p258) was also tested. The cell mixtures were pelleted, fixed in methacarn for 24hrs, then processed routinely into paraffin blocks. Four 10μm sections were taken from each block and boiled in 100μl of the lysis buffer (Method 3 section 2.3.1). Five μl of each of these DNA template preparations was used in separate standard dPCR reactions, incorporating primers for IFNG150 and c-erb-B2. Ratio values for each fixed cell mixture were calculated. The placental DNA p258 was used as a control for the normal copy number of one for both genes.

Differential PCR ratio values increased with increasing c-erb B2 copy number, a ratio value of 0.4 corresponded to 0.5 copies of c-erb B2 (MCF7) and a ratio value of 1.2 corresponded to approximately 6 copies of c-erb B2, see Table 10. These ratio values are lower than values expected, according to those found in Figure 16, however the ratio value obtained for control DNA p258 was within its normal range, suggesting that the PCR reaction dynamics were similar in both experiments. The apparently low ratio values and copy numbers in "amplified" mixtures may have been due to inaccuracies in cell counting, and copy numbers tested were below the effective sensitivity of the test. Nevertheless, fixation does not appear to artificially increase dPCR ratio values.

Table 10.

Differential PCR ratio values obtained from a titration of c-erb B2 gene copy number in DNA prepared from fixed cell pellets of MCF7 and UISO BCA1 tissue culture cells. Cells were mixed to give projected c-erb B2 copy numbers of 6,4, and 2. C-erb B2 copy numbers of 0.5 and 1 are from a fixed cell pellet of MCF7 and control DNA (p258) respectively. Ratio values for duplicate experiments are given.

Projected c-erb B2 copy number	**c-erb B2:IFNG ratio**	
	expt1	**expt2**
6	**1.2**	**1**
4	**0.9**	**0.79**
2	**0.7**	**0.65**
0.5 (MCF7)	**0.4**	**0.4**
1 (p258 fresh DNA)	**1.3**	**1.09**

2.3.5 Factors with Potential to Affect dPCR Ratio Values.

2.3.5.1 Assessment of Template DNA Degradation.

Degraded DNA may have the potential to distort dPCR ratio values, therefore dPCR with primer pairs IFNG150 and IFNG82 (both specific for the same gene, but different sequences) was used to investigate possible DNA degradation in methacarn fixed tissue specimens. The copy number should be the same for both amplicons. If there was degradation of the initial template DNA, such as may occur during fixation, the shorter amplicon of IFNG82 would be less likely to be disrupted, and therefore would appear to give an amplified value in the differential PCR reaction.

2.3.5.1.1 Validation of dPCR using Primers for IFNG150 and IFNG82. The reaction efficiencies of IFNG150 and IFNG82 were tested in dPCR by titration of IFNG82 primers with IFNG150 and throughout a thermal cycle course. IFNG82 primer concentrations of 75pmol 125pmol, 175pmol, and 250pmol were titrated against 250pmol of IFNG150 primer in a standard PCR reaction containing DNA template from control DNA(p258) and DNA from two normal breast tissue controls. Cycling parameters and calculation of ratio values were as stated previously for c-erb B2:IFNG150, section 2.2.3.2 and 3. A correction factor of 2.2 was applied to IFNG82 CPM to compensate for the smaller number of dCTP bases in its sequence, see Table 6. Radiation counts obtained for IFNG82 PCR products were divided by those for IFNG150 to give the final ratio value for this primer pair. Differential PCR ratio values obtained with different concentrations of IFNG82 primers showed some variation, but a general trend towards increasing dPCR ratio value with increasing IFNG82 primer concentration was apparent, Figure 17.

Figure 17

Titration of IFNG82 primers (82, 125, 160 and 250pmol) in a standard dPCR reaction containing 250pmol primers for IFNG150 for three control samples p258 (placental), alh490n and alh492n (normal fixed breast tissue).

Figure 18

Differential PCR ratio values for primers IFNG150 and IFNG82 throughout a thermal cycle course in a standard dPCR. PCR product was sampled at 20, 23, 25, 27, 32 and 35 cycles.

Figure 17 Titration of IFNG82 primers in dPCR

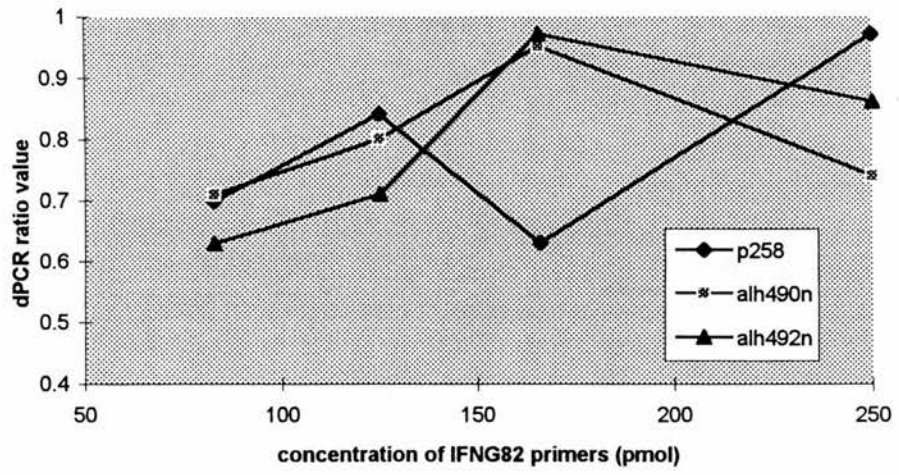
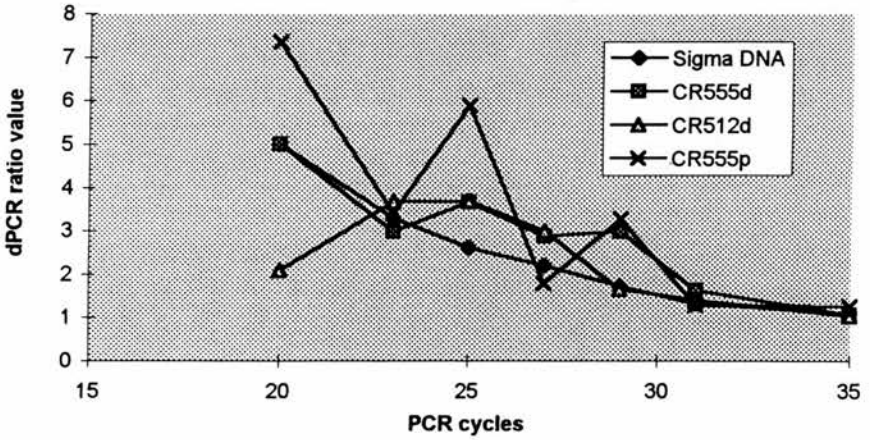


Figure 18 Differential PCR ratio values for IFNG150:IFNG82 throughout a cycle course



Primer efficiency was measured by sampling dPCR products throughout the progression of thermal cycles. The standard dPCR reaction was performed on control DNA (Sigma) and DNA from two breast cancers, as described above, except that total reaction volume was 200µl. Twenty µl of the PCR reaction was sampled at 20, 23, 25, 27, 29, 31 and 35 cycles, and PCR products electrophoresed and ratio values calculated as described in section 2.2.3.2. Sampling of dPCR products throughout the course of thermal cycling showed a steady decrease in ratio values with successive cycles, Figure 18, indicating that reaction efficiencies of these primer pairs are different.

2.3.5.1.2 Test Samples. Following this calibration, 5µl of prepared DNA suspension from 52 methacarn-fixed cancer samples and 37 fixed control tissues were tested in dPCR with primers 250pmol of primers IFNG150 and IFNG82, by the standard method and ratio values calculated (section 2.3.2). Ratio values ranged from 0.35 to 1.95 in control tissues and from 1.2 to 5.87 in cancer tissues; the distribution of these values is shown in Figure 19.

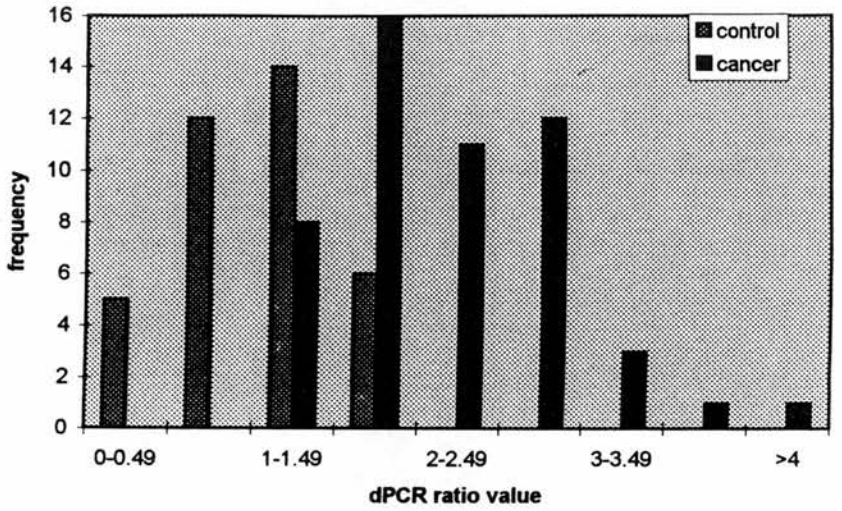
2.3.5.2 Assessment of the Integrity of the Reference gene, IFNG150, using dPCR with Primers IFNG150 and IFNB.

Differential PCR with primer pairs for IFNG150 and interferon beta (IFNB) assessed the likelihood of possible disregulation (amplification or loss of an allele) in the reference gene, as both should remain one copy. Primer efficiency of IFNG150 and IFNB was tested in the dPCR and optimised reaction conditions used on test samples.

Figure 19

Distribution of differential PCR ratio values for IFNG150 and IFNG82 in 52 breast cancers and 37 control tissues.

Figure 19 Distribution of IFNG150:IFNG82 dPCR ratio values in cancers and controls



2.3.5.2.1 Validation of dPCR using primers for IFNG150 and IFNB. IFNB primers concentrations of 31, 63, 125 and 250pmol were titrated against 250pmol of IFNG150 primers, containing DNA template derived from one control DNA (p258) or two DNAs from normal breast tissue, in a standard dPCR reaction as described 2.2.3.2. Ratio values were calculated as described in section 2.2.3.3. A correction factor of 1.27 was applied to CPM from IFNB, see Table 6. Titration of IFNB primers in the dPCR indicated an optimal concentration of 125pmol (1:2 dilution) of each IFNB primer with 250pmol of IFNG150 for subsequent dPCR, Table 11.

2.3.5.2.2 Test samples. Interferon gamma 150 reference gene status was then assessed in sections from 57 fixed breast cancer tissues and 27 control tissues, using 125pmol of each IFNB primer and 250pmol of each IFNG150 primer in each standard dPCR reaction. Ratio values for each tissue were calculated as described above. Ratio values ranged from 0.81 to 1.9 in breast cancers and from 0.4 to 1.7 in control DNAs. The distribution of ratio values found for each group is shown in Figure 20. Although the breast cancer group showed on average higher ratio values than controls, this difference was small (0.5 of a ratio value) and did not suggest significant disturbance to IFNG150 copy number.

Table 11.

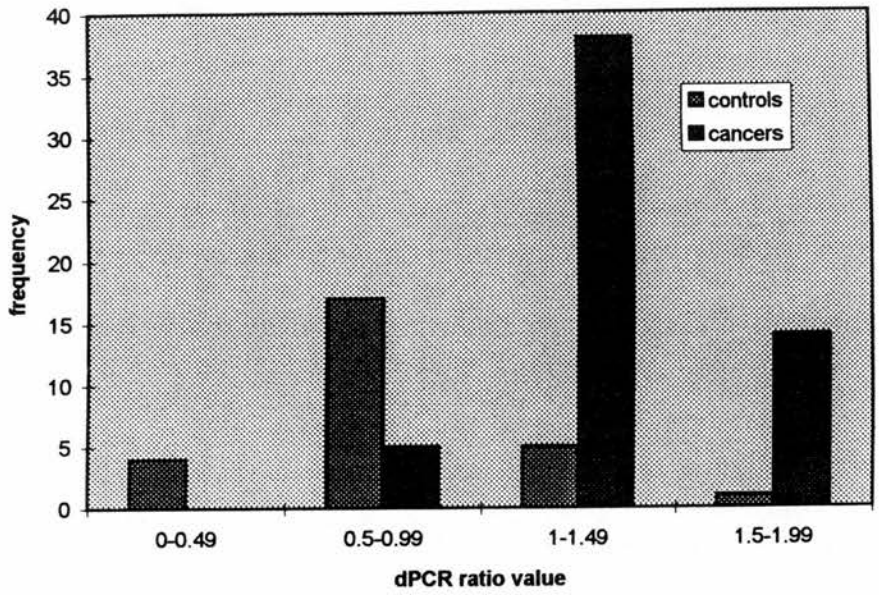
Differential PCR ratio values obtained from a titration of interferon beta (IFNB) primers with 250pmol interferon gamma 150 (IFNG150) in a standard dPCR reaction for one normal control DNAs, p258, and two fixed normal breast tissues ALH480n and ALH482n.

	Concentration of IFNB primers (pmol)			
Specimen	**250**	**125**	**63**	**31**
p258	**2.87**	**1.28**	**0.51**	**0.37**
ALH480n		**1.34**	**0.68**	**0.38**
ALH482n		**1.69**	**0.75**	**-**

Figure 20

Distribution of differential PCR ratio values for IFNG150 and IFNB in 57 breast cancers and 27 control tissues.

Figure 20 Distribution of IFNG150:IFNB dPCR ratio values in cancers and controls



2.4 DISCUSSION

The differential PCR has been proposed as an efficient method of measuring gene amplification in fixed, paraffin embedded tissues (Neubauer et al 1992). However detailed investigation of preparative methods for methacarn-fixed breast tissue, dPCR reaction dynamics, and the relationship between dPCR ratio values and gene copy number have not been previously reported. Experiments performed in this chapter investigated each of these parameters and optimised reaction conditions in order to apply the dPCR technique to clinical breast cancer samples, in a robust, reproducible manner.

Tissue fixation is thought to degrade DNA and effect molecular arrangement (Doyle and O'Leary 1992, Jackson et al 1990), and may limit molecular analysis. All breast cancer tissues in this study were fixed in methacarn, which has been shown to be an effective fixative for molecular studies (Greer et al 1990). Detailed testing of template DNA preparation methods produced a simple method of extracting DNA from methacarn fixed tissues which has significant improvements over previous methods. The preparation time was short (total approximately 30 min) and maintained the integrity of the template DNA so that it was resistant to several freeze-thaw cycles. DNA prepared from tissues fixed in methacarn produced similar results in dPCR to those of DNA prepared from fresh tissue, further indicating its suitability for this analysis.

Differential PCR is a highly complex and sensitive reaction and dynamics of the reaction may be effected by minor changes in reaction conditions. The key reaction in dPCR is thought to be the stability of the binding of primers to template DNA (Sugimoto et al 1993). For optimum dPCR, the two sets of primers should have

similar efficiencies (Sugimoto et al 1993), which can be demonstrated by detecting constant ratio values over a cycle course. This was clearly demonstrated for c-erb B2 and interferon gamma 150 gene primers (see Figure 15), indicating their validity in dPCR. This is also reflected in the clear correlation between the ratio values and gene copy numbers (see below).

In contrast, interferon gamma 150 and 82 gene primers showed dissimilar efficiencies (see Figure 18), resulting in changing dPCR ratio values with PCR cycle number and template DNA concentration. These primers appear to be of limited value in dPCR, yet they have been used to assess degradation of DNA prepared from fixed tissue (Neubauer et al 1992, Liu et al 1992). Experimentation with fractionated DNA has indicated that dPCR ratio values can be distorted when the average size of DNA fragments is less than 200 base pairs (Neubauer et al 1992). This degree of degradation resulted in IFNG150:IFNG82 ratio values of 3 or greater. On this basis twenty percent of breast cancers in one study were eliminated from further dPCR analysis (Liu et al 1992), perhaps creating an experimental bias. In this study, only 5 breast cancers tested in dPCR with primers for IFNG150:IFNG82 had ratio values over 3, and of these only 2 had amplified c-erb B2 ratio values. This provides additional evidence that dPCR with IFNG150:IFNG82 is not a robust and valid test. The measurement of DNA degradation in fixed tissue samples and its possible effects on dPCR therefore remain unresolved. It is possible that DNA degradation may be of only limited significance in most dPCR reactions (Neubauer et al 1992).

The relationship between dPCR ratio values and c-erb B2 gene copy number is not well documented. Rudimentary associations between cell lines of known c-erb B2 copy number and dPCR determined copy number have indicated a proportional relationship, but the ratio values obtained in dPCR have not been stated (Frye et al

1989, Neubauer et al 1992). In other studies, standard curves of c-erb B2 amplifications have been created by adding c-erb B2 amplicons to control DNA to mimic c-erb B2 amplified DNA (Lonn et al 1992, Lonn et al 1993). A linear relationship was demonstrated between ratio values and c-erb B2 copy number within the range of 2-10 copies, but higher amplifications proved difficult to measure (Lonn et al 1992). Accuracy in this type of assessment may be compromised by difficulties encountered when measuring the concentration of small DNA fragments. In this study a titration of c-erb B2 copy number was created using DNA from cell lines with known c-erb B2 gene amplifications, and mixing this in various proportions with control DNA to provide different c-erb B2 copy numbers. In agreement with Lonn et al (1992) a linear relationship was observed with low to medium c-erb B2 copy number, but became non-linear with high amplifications (greater than 32). This may result, in part, from variation in ratio values obtained in triplicate tests of highly amplified samples, see Figure 16. Differential PCR in highly amplified samples produces small amounts of reference gene product, therefore minor changes in product amount are likely to result in some variation in dPCR ratio values. However, these ratio values remain within the "highly amplified" range.

Other studies have reported difficulty in assessing the degree of large copy number amplifications in breast and ovarian cancer tissues (Hruza et al 1993, Lonn et al 1992, Liu et al 1992). One reason for this may be that they used laser densitometry to measure PCR product, and dense bands representing amplified genes may be outwith the linear range of the densitometer. Ultraviolet densitometry was assessed in this study, but demonstrated unacceptable inter-experimental variations in control values. It was therefore an inappropriate method for measurement of differential PCR ratio values. The incorporation of radio nucleotides into dPCR amplicons proved an accurate, numerical method for quantifying PCR product, which was not

limited by factors such as the sensitivity of densitometry. This enabled a wide range of c-erb B2 gene amplifications to be detected. Correction factors calculated to compensate for differences in dCTP bases, see Table 6, which can be labelled with ^{32}P, are important in determining PCR product equivalence. Calibration of dPCR ratio values with samples of known c-erb B2 gene copy number indicated that dPCR was not sensitive in detecting gene copy numbers between 2 and 5. However, while each ratio value was a derived number, proportional to the gene copy number, the tests outlined in this chapter indicate it is a reliable marker of gene amplification. The limits of accuracy of gene copy number determined by dPCR may change over the range of gene amplifications, where normal and low increases in gene copy number show little variation between duplicate tests, and high amplifications (discussed above) show greater variation, see Figure 17. For approximately 5 gene copies, ratio values ranged from 1.7 to 2.2, and suggests that there may be misclassification of some breast cancers. However, testing in duplicate should minimise this error. Amplifications of approximately 32 gene copies produced ratio values from 3.9 to 5.1, but even the lowest of these values would still be considered highly amplified.

Confusion has arisen in previous studies of oncogene amplification over the terminology used to describe an increase in gene copy numbers. The majority of gene amplification studies, either by Southern or Northern blotting, or by dPCR, refer to an amplification or increase in gene copy number, as a fold difference, e.g. a two fold increase, thus equating these to a copy number increase. The calibration of dPCR ratio values suggested that a ratio value of 2 is equivalent to 5 copies of c-erb B2. However the accuracy of this calibration is obviously dependent on the accuracy of the initial determination of gene copy number in the original cell lines, as determined by Southern blotting. As estimates of gene amplification from Southern blotting experiments are usually reported as a range, such as 2 to 4, this may

introduce some initial inaccuracy. I therefore decided to report some dPCR results as occurring in individual ranges (see Chapter 3) and where comparison with other studies is required, translated into low medium and high copy number.

A comparative assessment of the dPCR method outlined here, to previous methods of c-erb B2 gene copy number measurement indicates some major differences in how gene copy number was assessed and calculated. A complicated algorithm of four different dPCR reactions was designed to determine c-erb B2 gene copy numbers (Neubauer et al 1992). This algorithm included a test for DNA degradation, discussed above, and three dPCRs using primers for three different reference genes with primers for c-erb B2. Delimiting ratio values for each of these steps were not stated, and the number of samples which gave a negative result at each step were not declared. Amplifications appear to have been calculated according to the relationship of test dPCR ratio values to that of SK BR3, a cell line containing 4-8 copies of c-erb B2, and normal spleen. Using only these two gene copy references they claim a sensitivity of detecting twofold increase in gene copy number, clearly this must be very approximate. My direct assessment of ratio values was based on development of as complete a knowledge of dPCR reaction dynamics as possible.

In general, my testing indicated that measurement of c-erb B2 copy numbers by differential PCR is a valid technique for a wide range of experimental situations. Its strengths lye in the ability to determine c-erb B2 gene amplification in fixed paraffin embedded tissue, thereby allowing examination of a consecutive series or breast cancers. One weakness is in identifying cancers with low order gene amplification. I therefore proceeded to assess c-erb B2 gene copy number in a large series of clinical breast cancers.

CHAPTER 3

The Prevalence of c-erb B2 Disregulation in Breast Cancer

3.1 INTRODUCTION

Disregulation of the proto-oncogene c-erb B2 has been implicated in the aetiology of breast cancer (Slamon et al 1987). Although the gene was identified in 1981, (Shih et al 1981) we still know little of its normal biological function and its role in breast cancer. C-erb B2 protein shows close homology to epidermal growth hormone receptor and studies of c-erb B2 activity in rodents and cell lines have suggested a potential role in growth promotion (Chazin et al 1992, DiFiore et al 1987, Hudziak et al 1987, Yamamoto et al 1986). A number of possible receptor ligands have been identified (Holmes et al 1992, Lippman et al 1991), however the possible biological consequences of their binding to receptor remains unclear. To investigate a potential role for c-erb B2 in breast cancer it is important to ascertain the prevalence and form of its disregulation, and at what point of cancer development this could occur.

Recent reviewers have collated the results from over 50 studies of c-erb B2 gene amplification, mRNA production and protein overexpression (Perren 1991, Singleton & Strickler 1992). However there were major differences between studies in the frequency and association of c-erb B2 disregulation with histopathological features or prognosis, making its exact involvement in cancer development and progression difficult to determine, see Chapter 1 Tables 1, 2 and 3 . C-erb B2 protein overexpression, usually measured by immunohistochemistry, has been observed in 15-49% of ductal carcinomas of no special type (DCI NST) (Berger et al 1988, Thor et al 1989). Messenger RNA overproduction, detected by Northern blotting or *in situ* hybridisation techniques, ranged from 23-60% of DCI NST (Rio et al 1987, Walker et al 1989b), however numbers of cases in these studies tend to be small. Gene amplification measured by Southern or dot blotting has been observed in 10-

33% of DCI NST (Masuda et al 1987, Venter et al 1987). It is not clear whether these differences were due to variations in sample selection, study size, experimental technique or genuine biologically relevant disparity between populations.

This chapter extends the use of differential PCR, as assessed and validated in Chapter 2, to determine c-erb B2 gene amplification in a large consecutive series of breast cancers. Cancer characteristics such as DNA ploidy and cancer cellularity were examined for their relationship to dPCR ratio values. Disregulation of c- erb B2 is examined at three possible stages of disfunction; gene amplification, mRNA production and protein expression. How these events could interrelate and possible biological effects and consequences on cancer cells are discussed.

3.2 MATERIALS AND METHODS

3.2.1 Study Set.

The study tissues (314 cases) were collected from primary operable (clinical stage I and II) breast cancers at routine operations which included mastectomy and excisional biopsy both for palpable and non palpable lesions. Samples were mainly restricted to cancers of patients in the age group 50-65, collected between January 1988 and May 1990. Cancer tissues were fixed in methacarn (6:3:1 methanol:chloroform: acetic acid) overnight at 4^{o}C, processed according to routine methods and embedded in paraffin. Control tissues (43 cases) were obtained from breast tissue distant to the lesion site or from non-cancer bearing breasts and processed similarly.

Pathological characterisation was taken from an overall evaluation of material used for routine diagnosis. I cut, stained and evaluated a 4μm section (H and E stain) immediately adjacent to sections taken for dPCR (see below). Examples of stained sections confirmed the nature of the tissue used in the PCR reaction. The cellularity of each stained section was assessed subjectively for the proportion of the cancer cellular content. Cellularity was designated as either; 1=more than 75% , 2= 25%-75%, or 3= < 25%. In 26 cases (all breast cancers), samples of the lesion were taken and stored frozen in liquid nitrogen for RNA analysis.

3.2.2 Determination of DNA Ploidy by Flow Cytometric Analysis.

Two hundred and thirty five cancers were processed for DNA flow cytometry for ploidy analysis, according to the method of Hedley et al (1983). Briefly, two 50μm sections were dewaxed using two changes of xylene and rehydrated. Each tissue sample was incubated for 30 minutes at 37°C in 0.5% pepsin (Sigma) in 0.9% saline adjusted to pH 1.5 with 2N HCl, then stained with 0.1% propidium iodide containing 0.004% RNAase. Isolated nuclei were counted and analysed using an EPICS C flow cytometer (Coulter Electronics Ltd, Hileah, Florida). Ten thousand nuclei were counted at 480nm excitation and the coefficient of variation calculated using STATPACK software (Coulter Electronics Ltd). Ploidy was assessed as either diploid (DNA index DI, between 0.9 and 1.10) or aneuploid (DI >1.10 and <1.90 or > 2.10). Tetraploids were classified as DI between 1.90 and 2.10 with more than 20% of the cells apparently in G2 plus M phase of the cell cycle. For inclusion the coefficient of variation for the peak value had to be less than 8%.

3.2.3 Detection of c-erb B2 Protein Expression by Immunohistochemistry.

Overexpression of c-erb B2 protein p185 in cancer tissues was ascertained using the rabbit polyclonal antibody , 21N, to this protein (Gullick et al 1987). A separate 4μ m section of each tissue was dried at 56°C then stained in a three stage peroxidase-antiperoxidase technique (Sternberger et al 1986). The primary antibody, 21N was used at a concentration of 3.3μg/ml in 0.1M Tris buffered saline (pH 7.6) containing 5% normal swine serum. Each section was incubated at room temperature for 90 minutes. Endogenous peroxidase was blocked by exposure to 1% hydrogen peroxide in methanol for 30 minutes before staining. Overexpression of c-erb B2 protein was defined as the presence of brown staining of surface membrane of cancer

cells. To score positive, more than 10% of cells had to show moderate to strong staining. Controls included a known positive case and a negative control employing a pre-incubation of the antibody with its corresponding peptide (1mg ml-1).

3.2.4 Quantification of c-erb B2 mRNA

Messenger RNA was extracted from the frozen samples and analysed by a standard northern blot method (Thompson et al 1990). Twenty μg of each total tissue RNA was denatured with formamide and formaldehyde at 55°C for 20 mins and RNA species separated by electrophoresis on a 1.1% agarose gel. The RNA was transferred to a nylon filter (Hybond-N, Amersham, UK) by the capillary action of 10x SSC (1.5M NaCl, 150mM Trisodium Citrate) and covalently fixed to the membrane filter using a UV transilluminator. To detect c-erb B2 mRNA, the filters were hybridised with a radio labelled RNA probe #107, a 1.7kb fragment of v-erb B (Semba et al 1985), according to the method of Church and Gilbert (1984). Filters were then washed to remove non-specifically attached probe and exposed to pre-flashed Kodak XAR film at -70°C. Filters were stripped and reprobed with a radio labelled alpha actin RNA probe (Minty et al 1981) as an internal control for loading. The degree of hybridisation of each radio labelled probe to the mRNA species was estimated by reading of the x-ray films by light densitometry. Results were expressed with respect to hybridisation to the actin probe.

3.2.5 Measurement of c-erb B2 Gene Copy Number by Differential PCR

3.2.5.1 Sample preparation. DNA for PCR analysis was prepared from fixed paraffin embedded tissue as described in Chapter 2 (section 2.2.2.4). Control DNA was derived from normal human placenta (Chapter 2, section 2.2.1.2).

3.2.5.2 Differential PCR. The standard differential PCR reaction was performed as described in Chapter 2 (section 2.2.3) with primers for DNA sequences specific for Interferon gamma (IFNG150) and c-erb B2. All specimens were assessed in duplicate experiments, both as duplicate gel tracks from each PCR tube and in separate repeat experiments. A negative control, 200ng placental DNA, and a blank reaction, containing no template DNA, was included in each batch of PCR reactions. PCR products were quantified according to the methods described in Chapter 2 (section 2.2.4), and expressed as ratio values.

3.3 RESULTS

3.3.1 C-erb B2 Amplification and Overexpression in Breast Cancers.

3.3.1.1 Gene amplification determined by differential PCR. 314 breast cancer specimens and 43 controls were tested for c-erb B2 amplification using primers for c-erb B2 and IFNG150. Representative differential PCR products from three breast cancers and a normal placental control DNA are shown in Figure 21. Differential increase of c-erb B2 products indicating amplification is illustrated, with corresponding ratio values of 1.3, 2.1, 3.5 (tracks 1-3) and 1.1 for control placental DNA (track 5). Template DNAs for PCR in tracks 3 and 4 were derived from fixed and fresh tissue from the same cancer, and demonstrate a similar ratio value, 3.5 and 3.6 respectively.

The ratio values obtained using primers for c-erb B2 and IFNG150 from both normal (n=43) and cancer tissues (n=314) are shown in Figure 22. The ratio values for normal tissues fell consistently between 0.6 and 1.9 (mean 1.2, S.D. 0.36). Ratio values of 2 or above were therefore considered to signify c-erb B2 gene amplification. A value of 2 corresponds to approximately 5 gene copies (see Figure 16, Chapter 2), and indicates that dPCR, in its present form , is unsuitable for exact specification of those cases with low copy number (<5). For some analyses gene amplifications were classified as low-medium copy number (ratio values 2-3 corresponding to approximately 5-10 gene copies) and high copy number (ratio values greater than 3, corresponding to greater than 10 copies). Duplicate experiments gave consistent results for cancers without gene amplification and those with gene amplifications of low-medium and high copy number.

Figure 21

Differential PCR products from IFNG150 (150bp) and c-erb B2 (98bp) size separated on a 2% agarose gel. Lanes are: A BRL molecular weight marker V; 1,2 and 3 three different cancers; 4 DNA prepared from frozen tissue from the same cancer as track 3; 5 normal control DNA(p258). Differential PCR ratio values for tracks 1-5 are 1.3, 2.1, 3.5, 3.6 and 1.1 respectively. Tracks 2,3 and 4 all show clear amplification of c- erb B2 product.

Figure 21

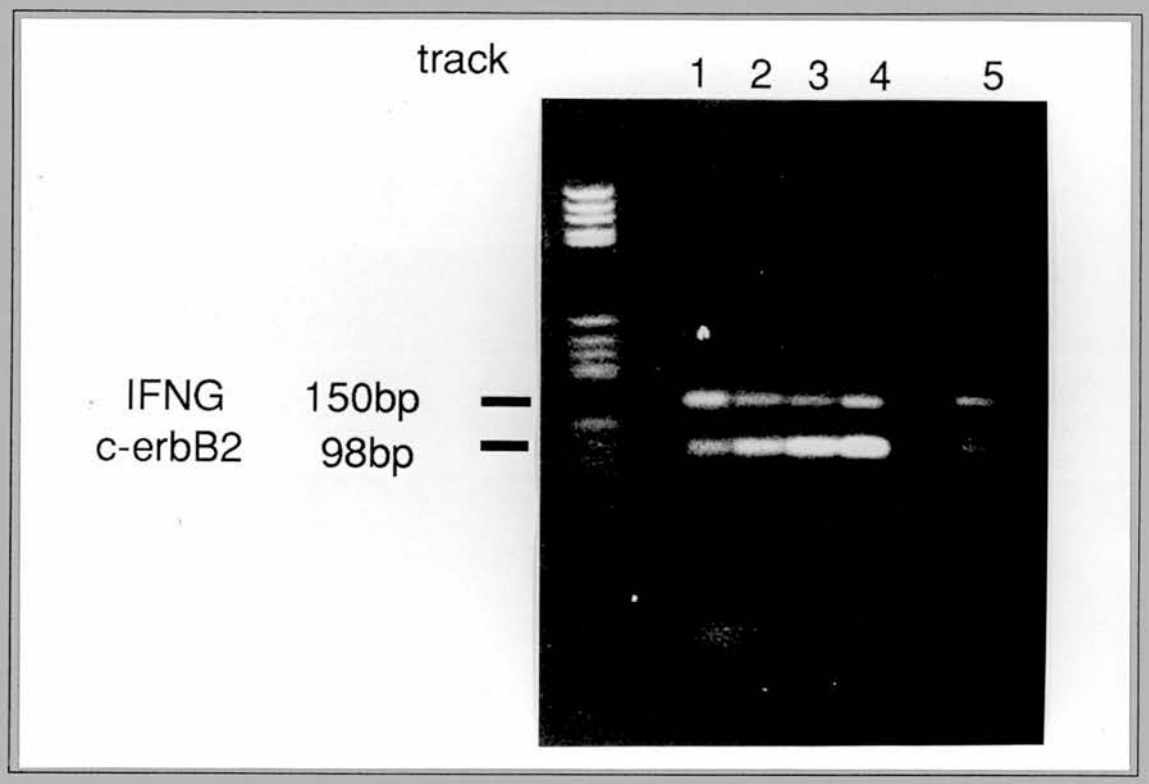
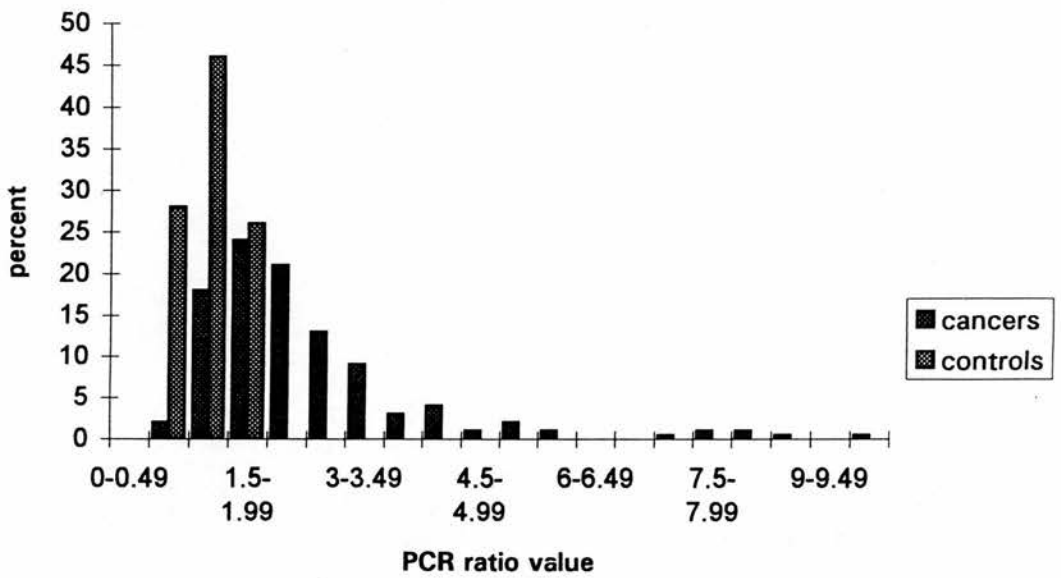


Figure 22

Distribution of differential PCR ratio values for c-erb B2 in 314 cancer tissues and 43 normal control tissues. Figures in columns are expressed as percent of cancers or percent of normal controls.

Figure 22 Distribution of c-erb B2 dPCR ratio values for breast cancers and controls



Cancer tissues had ratio values ranging from 0.6 to 19.2, Figure 22, indicating copy numbers throughout my calibration range (1 to 40). Fifty six percent of the total study set (n=177) had dPCR ratio values of 2 or above. For 41 *in situ* cancers the ratio values ranged from 1.0 to 8, with ratio values >=2 in 28 (68%), of which 17 (41%) were of low-medium and 11 (27%) were of high copy number. For 273 invasive breast cancers, the ratio values ranged from 0.6 to 19.2, with ratio values >= 2 in 149 (54%), corresponding to low-medium copy number in 93 (33%) and high copy number in 56 (21%). The difference in frequency of c- erb B2 gene amplification between *in situ* and invasive cancers was not significant, $X^2=2.73$ p= 0.10, Table 12.

3.3.1.2 Tissue localisation of c-erb B2 amplification. In order to confirm that gene amplifications were present in cancer cells but not in normal breast tissue dPCR ratio values were compared between cancer and normal tissue from the same tissue section. Localisation of c-erb B2 gene amplification was examined in a small number (n=3) of cancers in which normal tissue and cancer tissue were present in discrete portions within one paraffin section. This allowed dissection of the section into normal and invasive portions, and in one case an additional separate *in situ* cancer portion. For each component part, three 10μm sections were cut from each block and sub-divided using fresh sterile instruments. Portions of sections not taken for PCR were stained with haematoxylin and eosin to determine the accuracy of subdivision. Template DNA was prepared, (Chapter 2 section 2.2.2), and differential PCR performed, (Chapter 2 section 2.2.3).

Table 12

C-erb B2 amplification and cancer type. Cancers with dPCR ratio values of 2 or more were considered amplified. The difference in amplification frequency between in situ and invasive cancers was not significant, $X^2 = 2.73$ $p = 0.10$.

	c-erb B2 amplification	
	amplified	non amplified
in situ	**28**	**13**
invasive	**149**	**124**

Table 13

Differential PCR analysis of c-erb B2 gene amplification in normal and cancer tissue from three breast cancer cases.

	dPCR ratio value		
Case	**Normal**	**Invasive**	**In situ**
CR80	**2.13**	**2.55**	
CR832	**1.71**	**2.22**	**5.75**
CR868	**1.1**	**1.46**	

Differential PCR ratio values from dissected paraffin sections of three cancers are given in Table 13. No other cases with c-erb B2 gene amplification were suitable for dissection. Specimen CR832 has a higher degree of amplification in its *in situ* component than in invasive or normal parts. The high value for normal tissue from CR80 is possibly due to some contamination with cancer cells.

3.3.2 Cancer Characteristics which may Affect dPCR Ratio Values.

Differential PCR ratio values may be affected by a number of intrinsic features of breast cancers. Technical factors were discussed in Chapter 2, however biological factors such as DNA ploidy and cancer cellularity also have the potential to disturb dPCR ratio values.

3.3.2.1 DNA Ploidy. Satisfactory analysis of DNA ploidy by flow cytometry was obtained from 235 cancers. 112 had a diploid phenotype, and 123 were aneuploid or tetraploid. The frequency of amplification of c-erb B2 in specimens assessed by flow cytometry was found to be highest in cancers which were diploid (61%), with lower percentages of aneuploid (47%) and tetraploid (49%) cancers being amplified. These differences, however, were not statistically significant, $X^2 = 3.43$, $p0.50 > p > 0.10$.

3.3.2.2 Cancer Cellularity. A second potentially confounding factor was the dilutional effect of normal cells present within the cancer tissue, perhaps reducing the detection frequency of amplification. The proportion of amplified and non-amplified cases of invasive cancer ranked according to section cancer cellularity is shown in Figure 23. Amplification was found in each of the groups, including those specimens where cancer cells constituted less than 25% of total cellularity.

3.3.3 Relationship of c-erb B2 gene amplification to gene function.

The biological effect of c-erb B2 gene amplification may be mediated via normal gene functions which involve the transcription of the gene to mRNA and translation to the receptor protein. It was therefore important to examine expression of c-erb B2 mRNA and receptor protein, and assess the inter-relationships between these and gene amplification.

3.3.3.1 Protein overexpression assessed by immunohistochemistry. Immunohistochemistry for c-erb B2 overexpression was performed on 328 breast cancer specimens. Overexpression of c-erb B2 was detected in 16 of 44 (36%) of carcinoma *in situ*, and in 36 of 284 (13%) invasive carcinomas, representing a significant difference, $X^2 = 15.89$, $p = <0.001$. In cases where *in situ* and invasive forms of cancer were present on the same slide no detectable differences in the staining pattern between them was observed. Staining was concentrated on epithelial cell membranes and stained cells were present evenly throughout the cancer (Figure 24), except in one cancer where focal staining of cancer cells was observed. Overexpression was not observed in normal epithelial or stromal cells.

Figure 23

Relationship between cancer cellularity and frequency of c-erb B2 amplification in 277 breast cancers. Cancer cellularity was assessed visually as >75% cancer cells =1, 25-75% cancer cells =2, < 25% =3. Specimens with a dPCR ratio value less than two are represented by dark columns, specimens considered to be amplified (dPCR ratio value of 2 or above) are represented by light columns.

Figure 25

Distribution of dPCR ratio values for immunohistochemistry positive (+ve) and negative (-ve) cancers.

Figure 23 C-erb B2 amplification and cancer cellularity

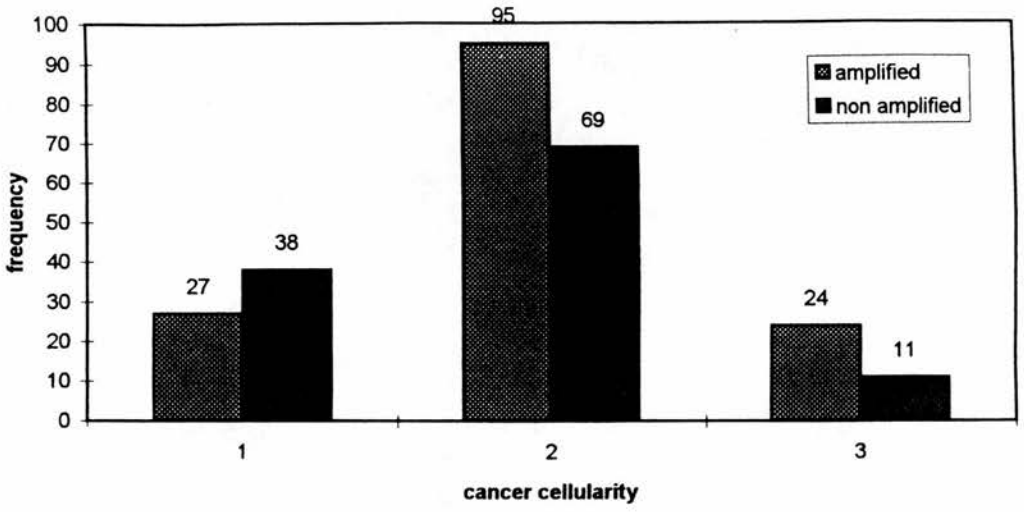


Figure 25 C-erb B2 amplification in with c-erb B2 protein expression

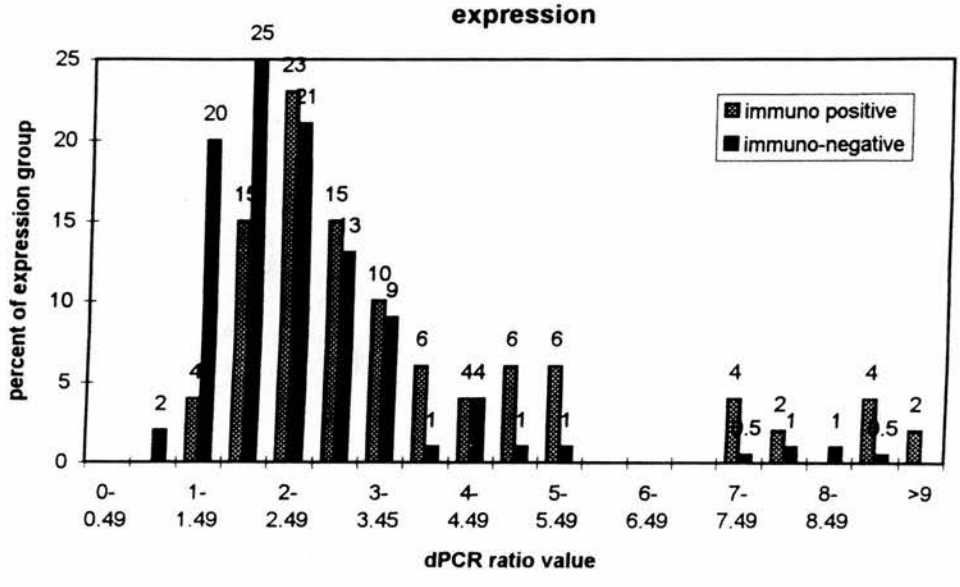
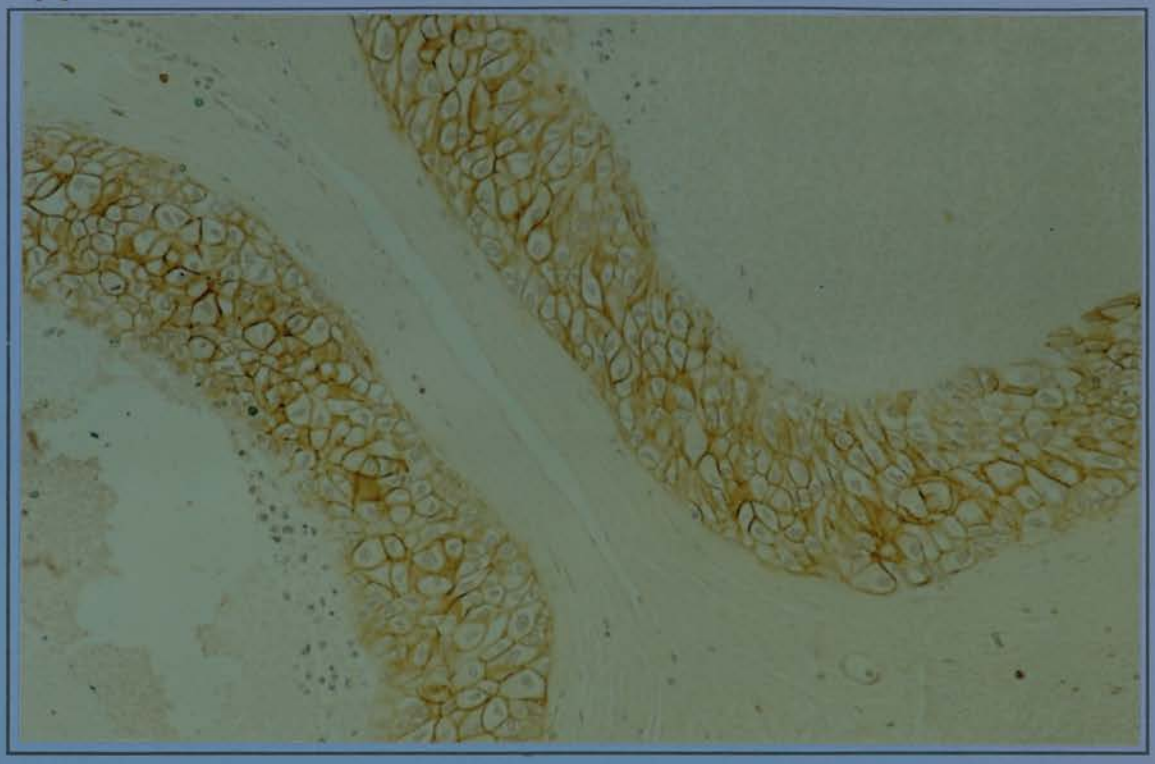


Figure 24

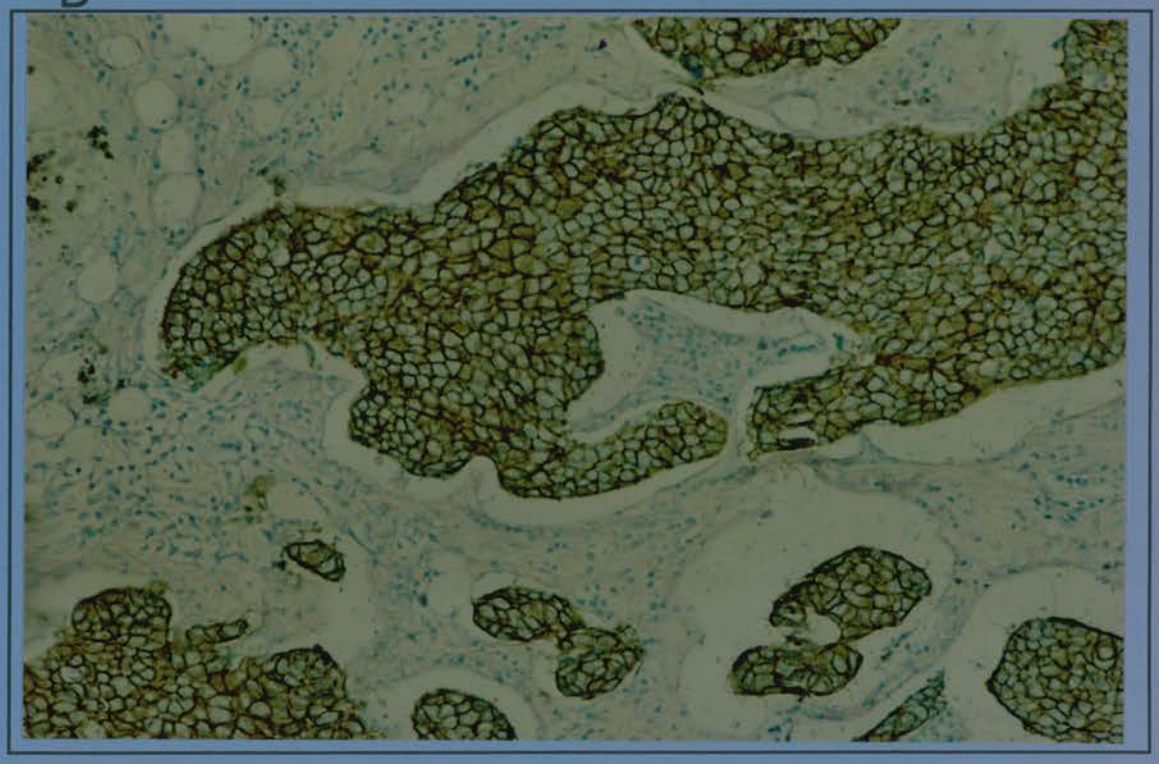
Photograph of a comedo type *in situ* carcinoma (A) and an invasive ductal carcinoma of no special type (B) stained with c-erb B2 specific polyclonal antibody, 21N. Cancer B shows fixation retraction of tissue. Staining, indicated by strong brown colour, was restricted to cancer cells and showed localisation to the cell membrane.

Figure 24

A



B



3.3.3.2 Comparative evaluation of protein overexpression and gene amplification. A case comparison of gene amplification determined by dPCR with protein expression determined by immunohistochemistry is shown in Table 14. Forty two of 51 immunopositive cases (82%) had gene amplification (with ratio values ranging from 2.0 to 19.2). There were 9 cases where differential PCR did not detect gene amplification in the presence of protein overexpression. However 135 of 263 immunonegative cases (51%) had PCR-detectable amplification of c-erb B2 gene, and this included 46 cases with ratio values >3, indicating a relatively high copy number. The range of differential PCR values was similar between the immunopositive and immunonegative groups (Figure 25), and applied to both *in situ* and invasive cancers. Of the 14 samples assessed by immunohistochemistry but not available for PCR, 1 was immuno-positive.

3.3.3.3 mRNA overexpression and c-erb B2 gene amplification and protein overexpression. Overexpression of c-erb B2 mRNA was identified by the presence of a positive hybridisation signal. The expression of c-erb B2 mRNA in normal tissue was not detectable by northern blotting techniques. Increased levels of c-erb B2 mRNA were found in 11 of 26 cases (42%). Correlation of the c-erb B2 mRNA values with gene amplification and overexpression status is shown in Table 15. All cases with positive immunohistochemistry contained elevated levels of c-erb B2 mRNA. Furthermore four of nineteen cases negative for immunohistochemistry also had elevated levels of mRNA; gene amplification determined by dPCR was present in two of these cases.

Table 14

Comparison of c-erb B2 protein overexpression measured by immunohistochemistry and c-erb B2 gene amplification measured by dPCR in 314 breast cancers, for an amplification delimitation values of 2. The overall frequency of amplification is 56%, and overexpression 15%.

	Overexpression	**No overexpression**
Amplification	**42**	**135**
No amplification	**9**	**128**

Table 15

Association of increased c-erb B2 mRNA expression with protein overexpression and gene amplification. All cancers with protein overexpression show elevated levels of mRNA. Four of 19 cases which were negative for protein overexpression have increased mRNA. Messenger RNA level appears to be independent of gene amplification.

	Immunohistochemistry			
	Positive		**Negative**	
mRNA	**positive**	**negative**	**positive**	**negative**
Amplified	**4**	**0**	**2**	**10**
Non-amplified	**3**	**0**	**2**	**5**

3.4 DISCUSSION

In this study c-erb B2 gene amplification was detected by dPCR in 55% of invasive ductal carcinomas (Table 12), a considerably higher percentage than previous reports employing other methods of detection of c-erb B2 gene amplification in breast cancers (Singleton & Strickler 1992). Most cases of gene amplification in both *in situ* and invasive cancers in this study, were in the lower range of amplification number. Amplification was often present without detectable c-erb B2 protein expression, but was rarely absent when protein overexpression was present. An accurate determination of gene amplification and its relationship to gene expression is important for a valid interpretation of the potential role and mechanisms of disregulation of c-erb B2 in breast cancer.

In this study the overall frequency of c-erb B2 gene amplification was high, with 56% of the total study set having increased gene copies. In another ten major studies of breast cancer, each assessing 100 or more cancer cases by Southern blotting, the frequency of detection of c-erb B2 gene amplification was consistently between 17% and 23% of the total study sets (reviewed in Singleton and Strickler 1992). However, in other studies, also using Southern blotting (Venter et al 1987, Andersen et al 1994) the percentage of cancers showing c-erb B2 amplification has been 33 to 70%. The direct comparison of amplification frequencies obtained by dPCR presented here and those in other studies is complicated by the incomplete knowledge of the relative sensitivities of the techniques and by differences in criteria and nomenclature used in assessment of increased gene copy number (see Chapter 2 for a discussion of gene copy nomenclature). Only one study of the relative sensitivity of dPCR or Southern blotting for detection of c-erb B2 gene amplification

has been reported (Hruza et al 1993). They found dPCR to provide reliable estimates of the numbers of extra copies in cases of gene amplification, similar to values obtained by blotting. They, along with other studies, also found that dPCR detected a higher percentage of cancers with gene amplification than Southern blotting methods (Hruza et al 1993, Imyanitov et al 1992, Slamon et al 1989, Zhang et al 1989). While other studies using Southern blotting have claimed to be able to detect very low levels of gene amplification, such as only two gene copies (Berns et al 1992, Borg et al 1991, Tavassoli et al 1989), these studies have lacked some validation requirements, such as the full range of values observed, the experimental variation in duplicate tests, cancer cellularity differences, and a control group of normal tissues. The dPCR as performed here would appear to lack the sensitivity to identify very low copy number increases of two to four copies.

In this study, the majority (61%) of c-erb B2 amplifications apparently involved only low to medium increases in gene copy number, of between five to ten copies, see 3.3.1.1. In another study, a similar percentage of amplified cancers also had low increases in gene copies (Borg et al 1991). It is not known if the number of gene copies is relevant to the behaviour of the cancer cell. Although it is generally assumed that c-erb B2 is expressed in proportion to the gene copy number, this has not been satisfactorily established, and is discussed below. Gene amplification may merely indicate a breakdown in cellular repair or control mechanisms. If so amplification of c-erb B2 would be a very frequent "random" event, perhaps maintained in the clonal population due to a selective growth advantage.

In this study, there were no clear differences between the frequencies of c-erb b2 amplification in *in situ* and invasive cancers, 68 and 54% respectively. Studies of c-

erb B2 amplification in *in situ* cancers have been restricted to dPCR, because their small size precludes Southern blotting analysis. Therefore the dPCR offers a great advantage in analysis of cancer progression involving *in situ* cancers. In another dPCR study of *in situ* and invasive cancers (Lui et al 1992), c-erb B2 amplification was detected in 48 and 21% respectively; however that study had a restricted study series and introduced a complex algorithm for dPCR calculations which excluded certain cases, as discussed in Chapter 2. Nevertheless, c-erb B2 amplification appears to be common in cancer types showing both invasive and non-invasive (*in situ*) phenotypes. It is therefore not valid to postulate c-erb B2 amplification as a marker for aggressive cancers. This is supported by studies relating case survival with the presence of c-erb B2 amplification (Ali et al 1988).

In this study, c-erb B2 amplification was often present without a detectable overexpression of c-erb B2 protein in the cancer cells. However, c-erb B2 amplification was rarely absent when protein overexpression was present. It is possible that the immunohistochemical methods employed in this study failed to adequately detect some overexpressing cancers. As mentioned above, the majority of cases in this study had low to medium amplifications whereas the antibody used will only detect expression in cells with at least 12 copies of c-erb B2 (Gusterson 1992). Detection of overexpression of c-erb B2 protein by immunohistochemistry is subject to considerable variation between studies (Singleton & Strickler,1993), due in part to differences in the primary antibodies, fixation methods, study set composition and criteria for assessment of staining.

There was a strong correlation between the presence of overexpression and amplification, eighty percent of immunopositive cancers had c-erb B2 gene amplification. Amplification was therefore rarely absent when overexpression was

present. Interestingly, there was a wide range of gene copy numbers in immunopositive cancers, see Figure 25. So some cases with strong immunostaining showed only a low increase in c-erb B2 gene copy number. It is possible that some enhancement of transcription and translation of the c-erb B2 gene occurs in some instances. Increased c-erb B2 mRNA levels, due to elevated amounts of a transcription factor, were observed in cancer cell lines with no detectable gene amplification (Hollywood and Hurst 1993). Other limitations of simple immunohistochemistry as a measure of disregulated gene activity have been recognised (Anderson 1992, Wynford-Thomas 1992). Detection of abnormal gene activity through *in situ* hybridisation methods (Kallionemi et al 1992, Smith et al 1993) may reveal more about the heterogeneity and degree of gene disregulation within cancer cell populations.

This variety of biological events and consequences suggest that a more realistic model to evaluate c-erb B2 disregulation in breast cancer must encompass a greater number of circumstances than (simply) gene amplification alone, and consider the interaction of other biological processes, see Chapter 8.

The presence of c-erb B2 amplification was not more apparent in aneuploid cancers. This indicates that the amplification was not a non-specific process associated with ploidy disturbances. This confirms the findings of previous studies (Lonn et al 1993, Ro et al 1989). An unexpectedly high frequency of amplification was found in cancers with a low cellularity of cancer cell to total cell numbers, see Figure 23. Testing of normal breast tissue did not produce any amplified c-erb B2 ratio values suggesting that some small cancers have high copy numbers of the c-erb B2 gene. Small cancers are not generally available for Southern blotting, due to insufficient tissue for DNA preparation, and were not included in another dPCR study (Lui et al

1992), therefore their amplification status has not been previously assessed. Cancer cellularity is only rarely taken into consideration in cancer gene studies (Slamon et al 1989). I performed a limited study on the localisation of gene amplifications within portions of a cancer containing separate invasive, *in situ* and normal elements which indicated that amplification levels are not always constant within a cancer.

A finding of "amplified" was based on a ratio value of 2 or above, as this was always above values obtained for control samples. Raising the cut-off point to a ratio value of 2.5 would reduce the number of cancers with "amplified" c-erb B2, but not change the relationships of c-erb B2 disregulations mentioned above. It is likely that the approximations and experimental variation inherent to other DNA analyses, including Southern or dot blotting techniques would also affect the precise amplification values obtained by those methods. The significance of c-erb B2 amplification may be merely that it is disregulated, not the exact number of gene copies.

Despite the problems of comparability, the dPCR technique has major potential to give a valid but different perspective of gene disregulation and cancer progression. To do this, I compared gene disregulation to factors such as cancer size, oestrogen receptor status, histopathological type and grade, lymph node status etc, see the following chapter.

CHAPTER 4

C-erb B2 Disregulation in Breast Cancer: Correlation with Cancer Characteristics.

4.1 INTRODUCTION.

Identification of the molecular events associated with progression in breast cancer is important to our understanding of the disease and for development of effective clinical treatments. The complexity of these associations is evident from the absence of any clear trends in the large number of studies to date. Some other cancers, such as colon cancer, are known to accumulate molecular disregulations as cancer progresses from adenoma to invasive carcinoma (Fearon and Vogelstein 1990). A similar accumulation of molecular events may occur in breast cancer, however the wide variety of histopathological forms and ill-defined precursor lesions have hampered their recognition. The likely progession from hyperplasia to *in situ* to an invasive cancer is widely accepted (Crisp et al 1993, Page et al 1982, Rosen et al 1980), but the scientific evidence to support this is difficult to acquire.

My study of cancers derived from mammographically screened breast cancers may provide a selection of cancers "caught" at an earlier stage of development than those detected symptomatically, and could therefore provide vital clues to early genetic events. Histopathological and biochemical features of breast cancer indicate considerable disease heterogeneity and are currently used in the estimation of cancer behaviour and as an aid to appropriate clinical treatment. Heterogeneity is illustrated by differences in features such as histological type, histological grade, lymph node metastasis and oestrogen receptor concentration (Perren 1991). Some of these features have an obvious chronological relationship with cancer progression, such as cancer size and lymph node metastasis, which usually increase over time. However oestrogen receptor status and cancer grade have a less well defined relationship with progression. In order to investigate the role of c-erb B2 disregulation in cancer progression, it is first necessary to consider the relationship between these other

features within the subgroups of the study set to provide a potential "framework" of cancer progression. Breast cancer type is determined by the pattern of cancer cell growth. Breast cancers commonly originate from epithelial cells which form the breast parenchymal units or lobular units (Bodian 1993). *In situ* carcinomas consist of cancerous cells which are still contained within the breast ducts and have not invaded surrounding stromal tissue. The presence of *in situ* carcinoma in many invasive lesions is suggestive of this being a precursor of invasive cancers (Black et al 1972, Page et al 1982, Rosen et al 1980). Special type cancers are invasive but generally of good prognosis (Dixon et al 1985), and may indicate that these histological types of cancer are less aggressive. These include tubular carcinomas, lobular carcinomas and mucinous carcinomas (Page and Anderson 1987). By far the most frequent type of breast cancer is invasive ductal carcinoma of no special type. These cancers show a more disorganised pattern of growth and exhibit considerable heterogeneity, but may still contain elements of *in situ* and or special type patterns of growth (Page and Anderson 1987).

Cancer grade is an important indicator of aggression or prognosis (Elston 1987). This method of classifying cancers, developed by Bloom and Richardson (1957), measures cancer cell differentiation status by assessing the presence of tubules formed by cancer cells, the extent of nuclear pleomorphism, and the number of mitoses present within a high-power microscopic field (Elston 1987).

Lymph nodes are commonly sampled at time of operation and histological examination is used as an indicator of cancer spread. The presence of cancer cells within the adjacent draining lymph nodes is likely to be the first indication of metastases, and is a clear indicator of poor prognosis (Clark et al 1991). The number of nodes involved and extent of node invasion may signify the stage of progression,

or chronological age of the cancer (Tubiana and Koscielny 1991). Terminal lesions are usually due to distant metastasis which may follow invasion of the lymph nodes (Burst and Ingold 1993). However, although lymphatic invasion does indicate one sort of progression, it may not be directly responsible for blood spread to bone, lungs etc (Burst and Ingold 1993). It is conceivable that cancers which metastasise to the lymph nodes are different to those which metastasise via the blood.

Oestrogen receptor (ER) concentration is measured in all suitable cancers for clinical treatment purposes. The presence of ER above 20 fmol/mg protein indicates that treatment with anti-oestrogens may inhibit tumour growth (Hawkins et al 1981). The exact role of ER in cancer progression is not clear, however the absence of ER in invasive cancers is an indicator of poor prognosis (Bernstein and Ross 1993). The functional role of oestrogen receptor in breast cancer remains unclear, but is likely to be multifactorial and involve the promotion of gene transcription (Dubik et al 1992, Hulka et al 1994). The presence of oestrogen receptors is thought to negatively correlate with c-erb B2 overexpression in cancer cells (Russell and Hung 1992), and suggests that oestrogen receptors may have a functional or regulatory relationship with c-erb B2.

This chapter aims to explore the inter-relationships between histopathological and biochemical features and biological measures of genetic disregulation in cancers from screened and symptomatic breast cancers and identify possible stages of progression in which disregulation of c-erb B2 may occur.

4.2 MATERIALS AND METHODS

4.2.1 Definitions of Breast Cancer Source

The study set consisted of breast cancers obtained, in a consecutive manner, from female patients the majority of whom were aged between 50 and 65. Cancers which presented symptomatically were referred to as never screened. Cancers which were detected by mammography were predominantly from new attenders to a prevalence round of the UK National Screening program during 1988-1990, and were referred to as newly screened. A small group of cancers were identified in women who attended or participated in a previous mammographic screening program (Roberts et al 1984) 1978-1988, and were referred to as previously screened.

4.2.2 Definitions of Breast Cancer Characteristics

Cancer size at the time of operation was noted for invasive cancers as the diameter of the lesion on macroscopic tissue examination. *In situ* cancers were not measured. Large inoperable cancers were allocated a nominal size of 51mm because accurate determination of size was not possible from their wedge biopsies. Lymph node status was noted in invasive cancers, as either positive or negative for the presence of malignant cells, following the histological examination of lymph node biopsies taken concurrently at surgery. Some breast cancers greater than 50mm in diameter did not have node samples taken as these formed part of a separate treatment trial (E. Anderson, Dept. Surgery, Univ. Edinburgh).

The histopathological type of all cancers were classified according the criteria of Page and Anderson (1987). The major cancer types identified in this study set are

illustrated in Figure 26 A-E, and listed in Table 16. Invasive cancers were graded for nuclear pleomorphism, tubule formation and mitotic rate, according to a modification (Elston 1987) of the method of Bloom and Richardson (1957). Each of these elements is given a score of 1 to 3, and summation of these gives a grading score: grade I (best prognosis) scores between 3 and 5, grade II 6 or 7, and grade III (worst prognosis) 8 or 9. *In situ* cancers were not graded.

4.2.3 Definition of Oestrogen Receptor Status.

Oestrogen receptors were measured by a radio-ligand binding assay using dextran coated charcoal according to the method of Hawkins et al (1981). The concentration of oestrogen receptors in each cancer sample was then expressed relative to the protein concentration in each sample. Values of >20fmol/mg protein have been defined as the minimum concentration of receptors necessary for clinical treatment with anti-estrogens (Hawkins et al 1981), and were therefore considered receptor positive.

4.2.4 Genetic and Biochemical Features of the Study Set

Methods for determination of DNA ploidy, c-erb B2 overexpression, c-erb B2 amplification and cancer cellularity were described in Chapter 3.

4.2.5 Data Analysis

Data points (field values) were entered into a Paradox for Windows database (Borland Ltd, USA). For each cancer the following fields were recorded: laboratory

number; date of birth; name; pathology reference number; screening status; lymph node status; cancer size; histopathological type; cancer grade; oestrogen receptor concentration; DNA ploidy status; c-erb B2 immunohistochemistry; and c-erb B2 amplification. Univariate analysis of field values for each of these groups were computed by Paradox software (Borland Ltd), and X^2 analysis of contingency tables applied to test significance. Results were formulated as frequencies or percentages of field values in comparisons between groups and were expressed graphically. Multivariate analysis was performed using logistic regression with analyses of deviance and the asymptotic chi-squared distribution for the log-likelihood to compare nested models. All multivariate analyses were implemented using computer software EGRET by Dr.F.Alexander.

Figure 26 A-E

Photomicrographs illustrating the main histopathological types of breast cancer:

A *In situ* ductal carcinoma. Cancer cell are contained within the duct, and can form different histopathological patterns, such as comedo (illustrated here), solid or micropapillary types.

B Invasive ductal carcinoma of no special type. This is the most common type of breast cancer. Cancer cells have invaded the normal tissue surrounding ducts and show no distinguishing histologic features.

Figure 26 A

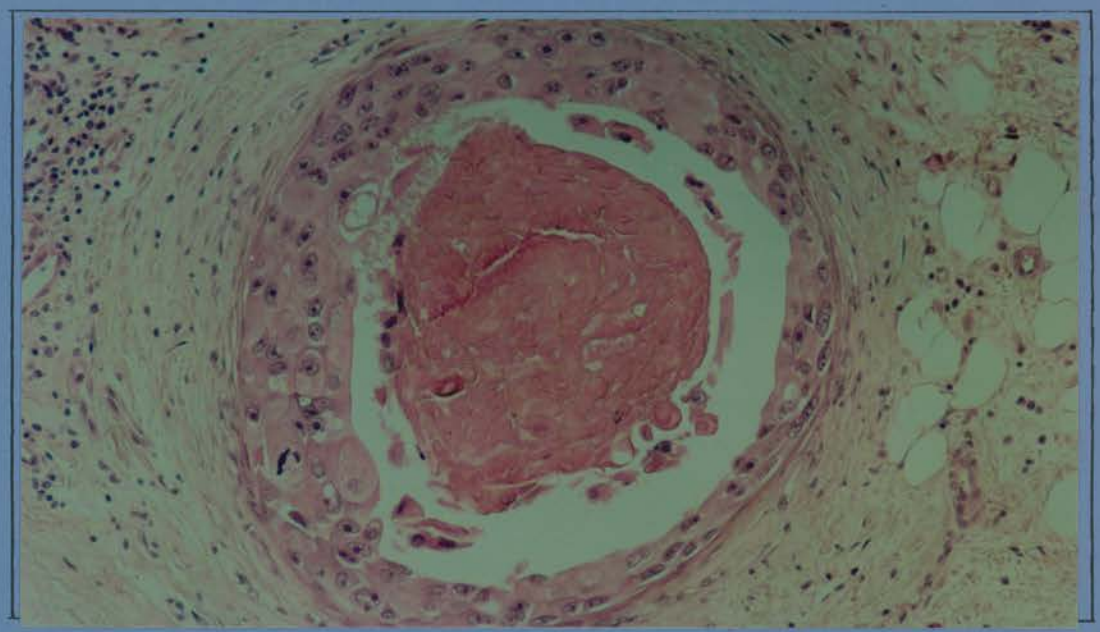


Figure 26 B

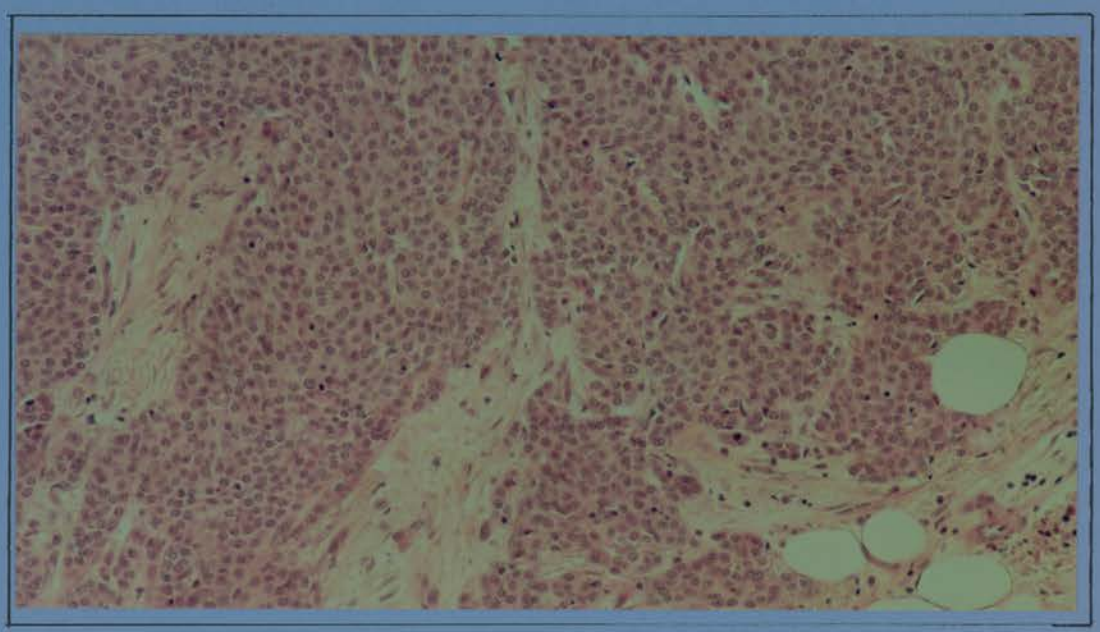


Figure 26 C-E

C Tubular carcinoma. Invasive carcinoma which forms tube like structures through normal breast tissues. Cancer cells are well differentiated and the epithelial tissue shows a high percentage of glands.

D Lobular carcinoma. Invasive carcinoma which forms chains of cells, usually in single file.

E Medullary carcinoma. Invasive carcinoma formed from solid sheets of large cells, with vesicular and polymorphic nuclei.

Figure 26 C

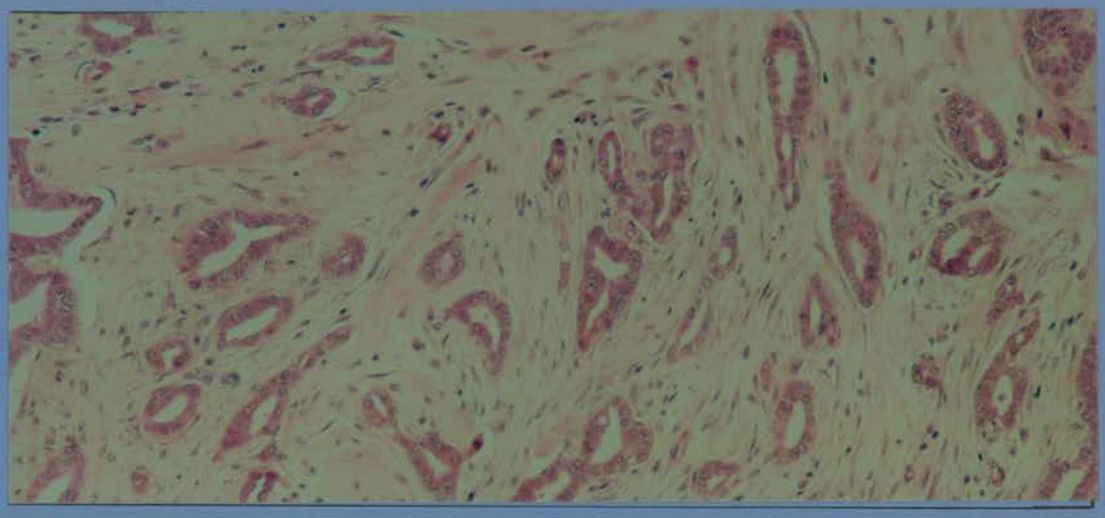


Figure 26 D

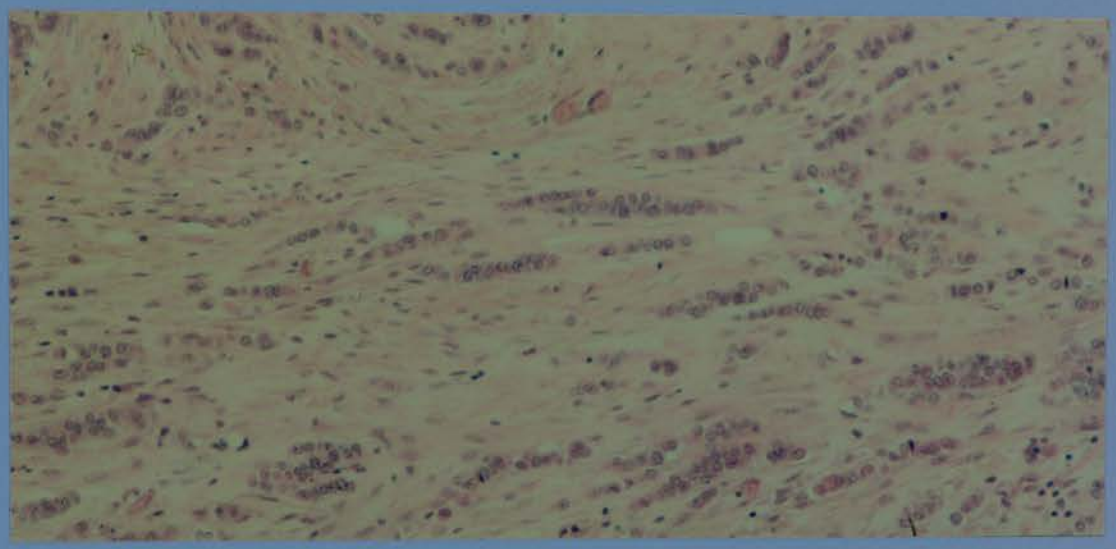


Figure 26 E

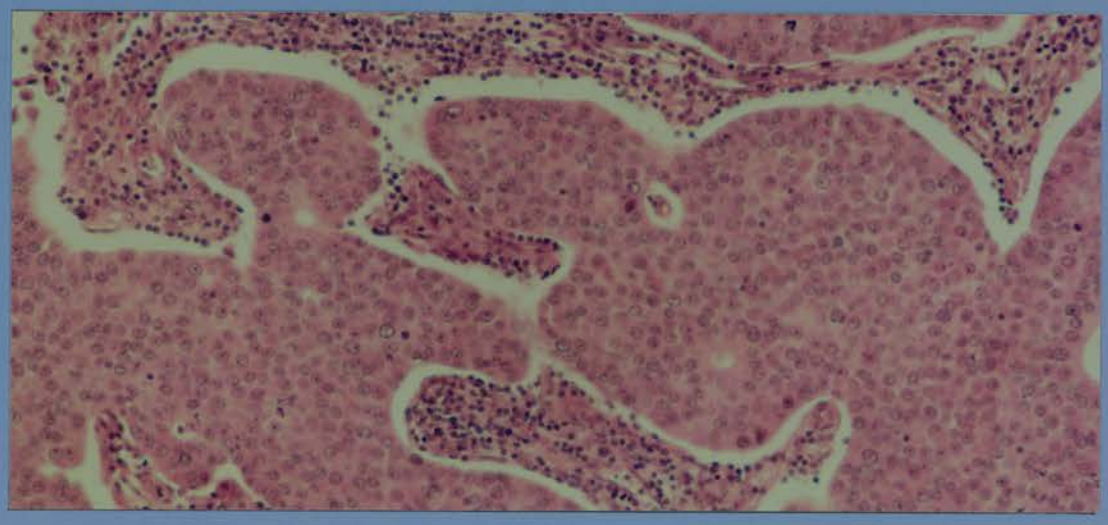


Table 16

This table lists the major histopathological types of breast cancer. The major classifications are *in situ* and invasive carcinomas, with subdivisions created by distinct pathologies within each group. The most common type of cancer is invasive ductal carcinoma of no special type.

Histopathological cancer type			
***In situ* carcinomas**	- Lobular		
	- Ductal	- comedo	
		- non comedo	micropapillary cribriform
Invasive carcinomas	- Ductal of no special type		
	- Special type	- Tubular	
		- Lobular	
		- Medullary	
		- Cribriform	
		- Others	

4.3 RESULTS

4.3.1 Characterisation of Breast Cancers

All cancers were classified according to histopathological type. *In situ* cancers, of ductal (n=42) and lobular origin (n=2), represented 14% of the study group. Seventy-four percent (n=244) of the study group were invasive ductal carcinoma of no special type. Of the special type cancers, lobular (n=20) and tubular (n=12) cancers were the most frequent, 6% and 4% respectively. Other special types medullary, and mucinous occurred infrequently, and together represented the remaining 2% of the total population. Two hundred and 51 invasive cancers were graded for nuclear pleomorphism, tubule formation and mitotic rate: grade I, n=64, grade II, n=143, and grade III (worst prognosis), n =44. Lymph node status was noted in 266 invasive cancers as either positive (n= 92) or negative (n= 174) for presence of malignant cells on histopathological examination.

4.3.2 Screening Status and Cancer Characteristics

The study set consisted of 334 breast cancers, of which 173 were never screened, 111 were newly screened and 44 were previously screened. Six cases had no screening status noted. Cancer characteristics were compared between cancers from the screening groups (previous and newly screened) and those in the never screened groups in order to ascertain if cancers in these groups could represent different stages of cancer development.

Screen detected (both previously and newly screened groups) invasive cancers show a significant trend towards smaller cancer size than never screened cancers, Figure 27, $X^2 = 42.46$, $p<0.001$. Thus 86% of the cancers in the 1-10mm size group are in the screening groups. This was not surprising as small cancers are less likely to be symptomatic. Only slightly reduced percentages of cancers >30mm in diameter occurred in the screening groups, suggesting the presence of some cancers which may have been symptomatic at time of detection. The excess of small cancers, although reduced, remained on analysis of ductal carcinoma of no special type only, $X^2 = 38.23$, $p<0.001$.

Lymph node invasion was present in each of the screening categories, with the highest frequency occurring in the previously screened group (41%), followed by never screened (34%) and newly screened (25%). These differences were not significant, $X^2 = 4.93$, $p<0.10$.

The distribution of cancer type was different between the newly, previously screened and never screened groups, $X^2 = 13.74$, $p<0.01$, Table 17. Invasive ductal carcinoma of no special type was the most frequent cancer type in each of the groups. Special type cancers, tubular and lobular, as well as *in situ* cancers were more frequent in the newly screened and previously screened groups than in the never screened group.

Grade I cancers were more frequent in the newly screened group (35%), than in the never screened group (22%), with grade III cancers showing the reverse trend, $X^2 = 11.4$, $p<0.025$, Figure 28. However, this difference was influenced by the presence of special type cancers in the screen detected cancers, and the test lost significance when DCI NST alone were analysed, $X^2 = 8.11$, $p<0.10$.

Figure 27

Distribution of cancer size (diameter in mm) for each screening group, previously screened, newly screened, and never screened. Cancer sizes have been grouped in 10mm size categories. Results are expressed as percentage of screening group.

Figure 28

Distribution of histopathological grade for each screening group, previously screened, newly screened, and never screened. Results are expressed as percentage of screening group.

Figure 27 Cancer size and Screening Status

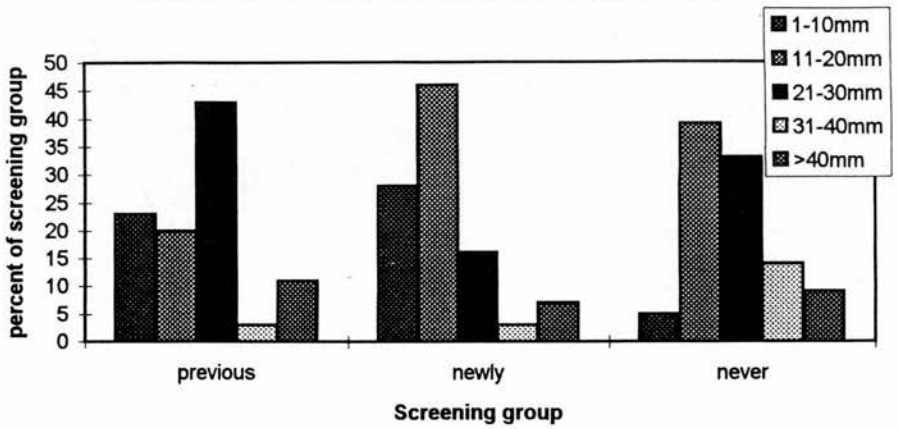


Figure 28 Cancer Grade and Screening Status

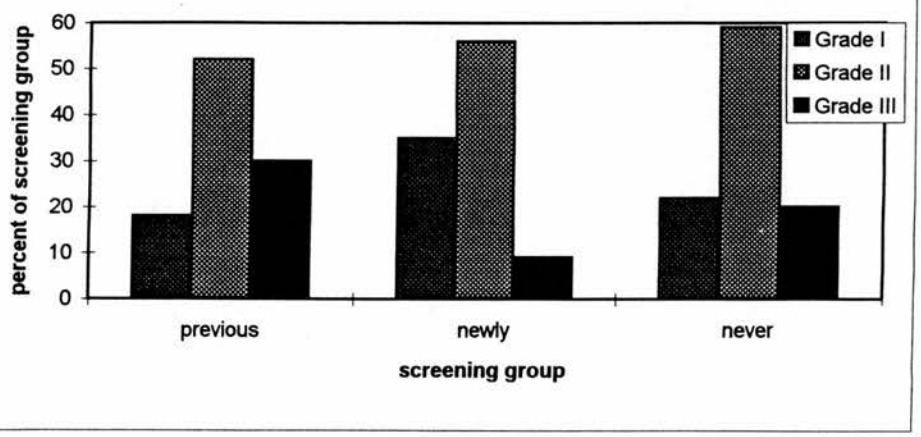


Table 17

Histopathological cancer types found in each of the screening groups, previously, newly and never, expressed as percentages of each group.

Table 17

Cancer type	previously	newly	never
in situ	**20**	**17**	**9**
Invasive			
No special type	**68**	**65**	**82**
Special type	**12**	**18**	**9**

Oestrogen receptor concentrations were divided into three groups <20fmol/mg protein, 20-200fmol/mg protein, and >200fmol/mg protein. An arbitrary value of >200fmol was chosen for more detailed assessment of oestrogen receptor associations. Newly screened group cancers were more likely to be oestrogen receptor positive (>= 20fmol/mg of protein), however the previously screened group show a higher percentage of ER negative cancers (37%) than either newly or never screened cancers, $X^2= 6.18$, $p<0.05$.

Never screened cancers had a greater frequency of abnormal DNA ploidy, aneuploidy (38%) and tetraploidy (21%), $X^2= 10.5$, $p<0.05$, Figure 29.

4.3.2.1 Summary of Cancer Characteristics of Screening Groups.

Differences in the distribution of cancer size, type and grade were observed between screen detected and never screened cancer groups. No difference in distribution of lymph node metastases, histopathological grade and oestrogen receptor status were observed. The frequency of some of these features, particularly histopathological type and grade, oestrogen receptor status, and DNA ploidy status can be influenced by the "length bias" inherent of the prevalence screen, in which a proportion of cancers are slow growing and indolent. Multivariate analysis (restricted to women aged 50-65) indicated that only cancer size and lymph node were chronological factors which changed over the time period of the screening program.

Some characteristics of the previously screened group were different to the newly screened group and indicated the presence of high grade, oestrogen receptor negative, and lymph node negative cancers, see Figure 28 and section 4.3.2. This

group was considered too small for statistical analysis, but suggests that interval or incidence cancers can have aggressive characteristics.

4.3.3 Inter Relationships between Histopathological and Biochemical Features of Breast Cancer.

The inter relationships between histopathological and biochemical features of breast cancer are complex. Cancer size and lymph node metastases are known as chronological features of breast cancer, and associations with other cancer features may provide an approximate "framework" of the particular features of cancers in the stages of progression.

Small cancers were correlated with lymph node negativity $X^2=12.64$, $p<0.01$ (Figure 30), low grade (grades I, $X^2= 21.75$, $p<0.01$, Figure 31), and less disturbance to DNA ploidy $X^2= 21.16$, $p<0.01$ (Figure 32) but not with oestrogen receptor status $X^2 = 6.71$, $p<0.10$ (Figure 33). Conversely large cancers were more likely to be high grade (grade III), lymph node positive, and oestrogen receptor negative. Disturbances to DNA ploidy did not appear to increase with cancer size in cancers over 20mm (Figure 32).

Lymph node involvement was present in several histopathological cancer types, but was not significantly different between ductal carcinomas of no special type and special type cancers, $X^{2=} 3.16$, $p<0.10$. Lymph node metastases were not associated with cancer grade X^2 0.48, $p=0.50$, oestrogen receptor concentrations, $X^{2=} 0.2$, $p=0.5$, or DNA ploidy disturbances $X^{2=} 0.26$, $p=0.5$.

Figure 29

Distribution of DNA ploidy status for each screening group, previously screened, newly screened, and never screened, expressed as a percentage of screening group. DIP = Diploid, AN = Aneuploid, TET = Tetraploid.

Figure 30

The association between lymph node status and cancer size (diameter in mm) in invasive cancers, expressed as a percentage of cancer size. Cancers were classified as node negative where there was no evidence of metastasis to the axillary lymph nodes, or node positive where cancer cells were present within the lymph node tissue. Lymph node positivity increases in a linear fashion with cancer size.

Figure 29 DNA Ploidy and Screening Status

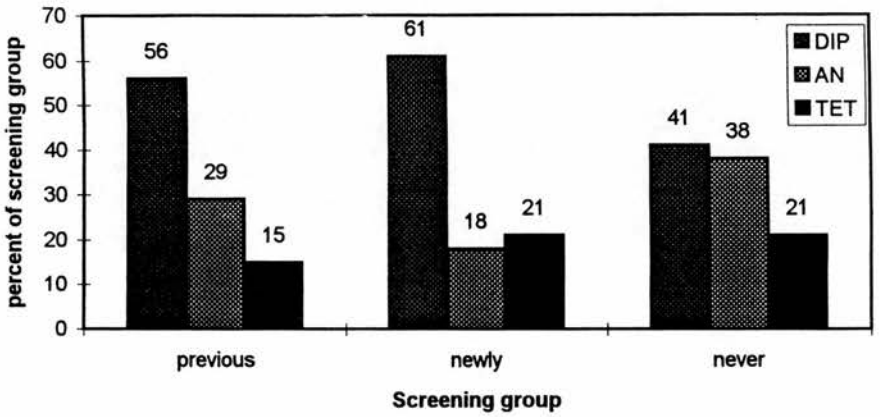


Figure 30 Lymph Node Status and Cancer Size

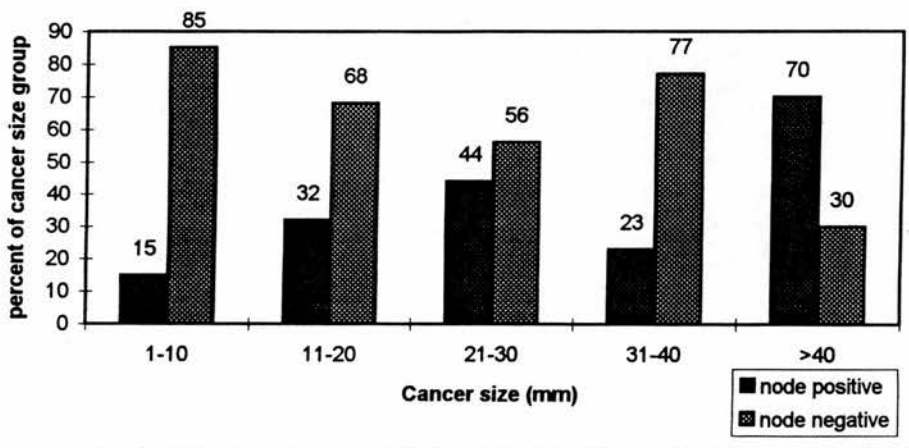


Figure 31

The distribution of histopathological grade with cancer size (diameter in mm), expressed as a percentage of size group. Cancer sizes have been grouped in 10mm categories. Grade I cancers decrease in frequency with increasing cncer size, and conversely grade III cancers increase in frequency with increasing size.

Figure 32

Distribution of DNA ploidy status with cancer size (diameter in mm), expressed as percentage of size group. Cancer sizes have been grouped in 10mm categories. DIP = Diploid, AN = Aneuploid, TET = Tetraploid.

Figure 31 Cancer Size and Cancer Grade

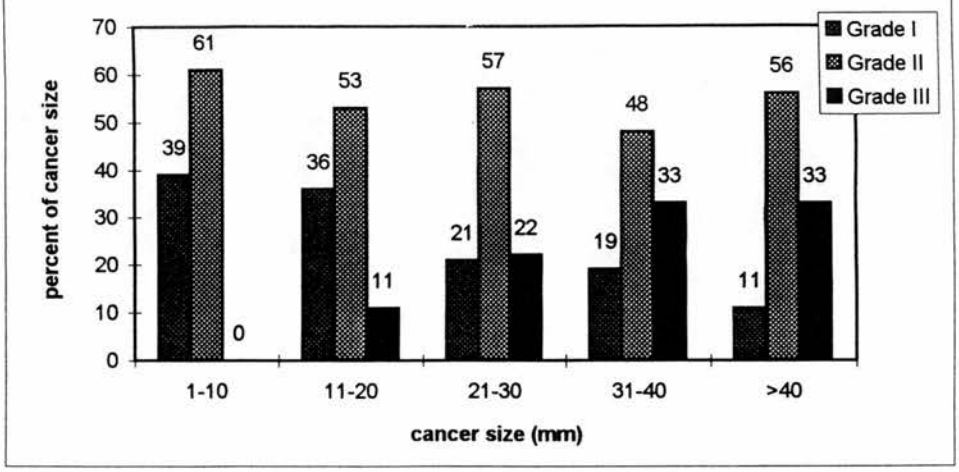
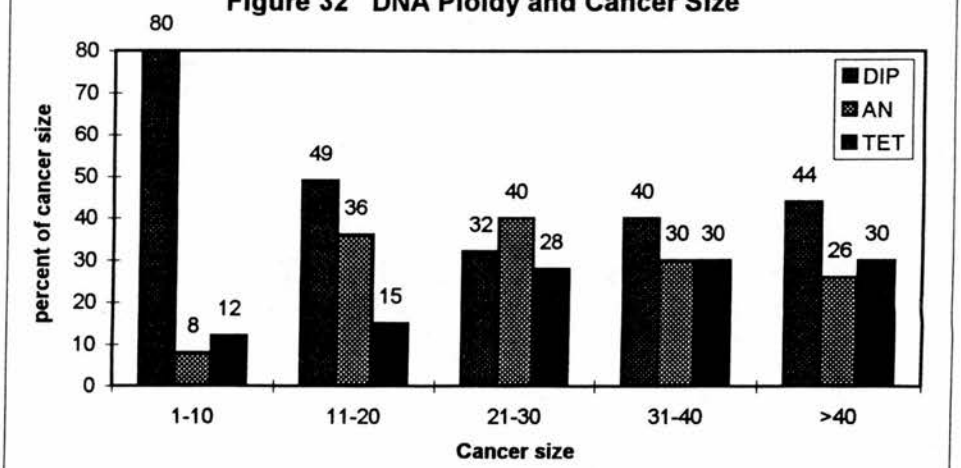


Figure 32 DNA Ploidy and Cancer Size



Other associations between biological characteristics may also be important for interpretation of cancer features in progression. Highly significant differences in cancer grade (X^2 = 27.1, $p<0.001$) and DNA ploidy (X^2= 24.91, $p<0.001$) were found between special type and no special type invasive cancers. Special type cancers were usually of low grade (grade I, Table 18), and showed less disturbance to DNA ploidy (Table 19).

Cancers which were negative for oestrogen receptor status (<20fmol/mg protein) correlated strongly with grade III cancers, X^2= 32.57, $p<0.001$ (Figure 34).

Relationships between these features help to provide a framework of chronological and biological features in cancer progression.

4.3.4 C-erb B2 Overexpression and Histopathological and Biological Characteristics of Breast Cancer.

In univariate analysis c-erb B2 expression was found to vary with cancer type, cancer grade and oestrogen receptor status, but was independent of lymph node status, screening status and DNA ploidy disturbances. Initial examination of expression in screen detected groups indicated that expression was more frequent in newly and previously screened groups, 16% and 23% respectively, than in the never screened groups (14%), Figure 35, but this was not a significant difference, X^2= 2.03, $p<0.50$, > 0.10. C-erb B2 overexpression was more frequent in cancers over 21mm in size, however the frequency did not increase with further increasing size, Figure 36, thereby resulting in a negative correlation, X^2= 7.53, $p<0.50$. This low frequency of

Figure 33

Distribution of oestrogen receptor status and cancer size (diameter in mm), expressed as percentage of size group. Cancer sizes have been grouped in 10mm categories. Cancers with <20fmol/mg oestrogen receptor (oestrogen receptor negative) increased in frequency with increasing cancer size.

Figure 34

The distribution of histopathological cancer grade with oestrogen receptor status, expressed as a percentage of cancer grade. Oestrogen receptor concentrations <20 fmol/mg protein were considered negative. The frequency of oestrogen receptor negative cancers increase with increasing cancer grade.

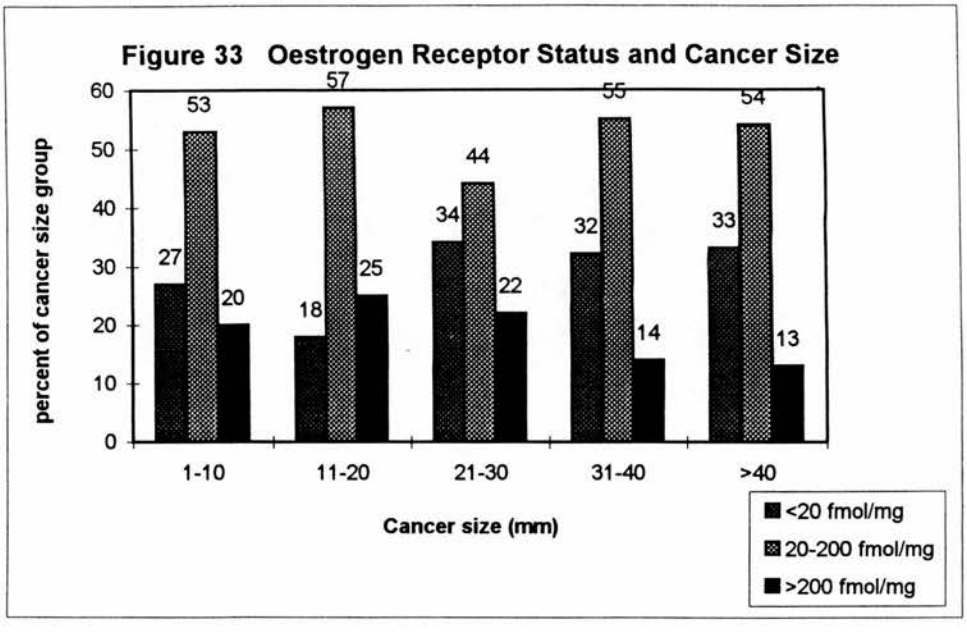


Figure 33 Oestrogen Receptor Status and Cancer Size
percent of cancer size group
60
50
40
30
20
10
0
27
53
20
18
57
25
34
44
22
32
55
14
33
54
13
1-10
11-20
21-30
31-40
>40
Cancer size (mm)
<20 fmol/mg
20-200 fmol/mg
>200 fmol/mg

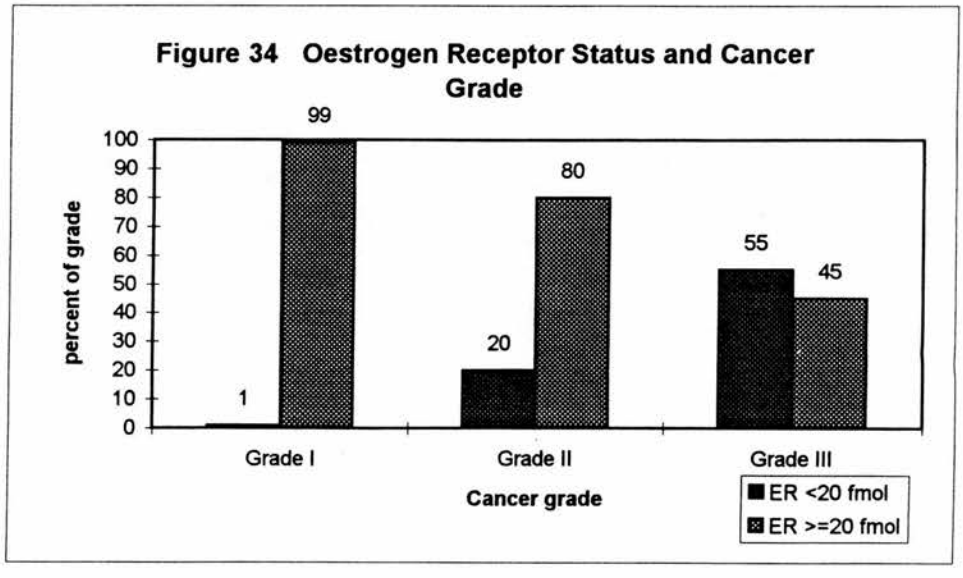


Figure 34 Oestrogen Receptor Status and Cancer Grade
percent of grade
100
90
80
70
60
50
40
30
20
10
0
1
99
20
80
55
45
Grade I
Grade II
Grade III
Cancer grade
ER <20 fmol
ER >=20 fmol

Table 18

Frequency of invasive cancer types with histopathological cancer grade.

Table 18

Cancer type	Cancer Grade I	II	III
No special type	**42**	**180**	**42**
Special type	**22**	**16**	**2**

Table 19

The frequency of histopathological cancer type with DNA ploidy status.

Table 19

Cancer type	Ploidy status Diploid	Aneuploid	Tetraploid
in situ	**20**	**6**	**4**
Invasive			
No special type	**77**	**63**	**40**
Special type	**20**	**5**	**5**

Figure 35

C-erb B2 expression and screening status, expressed as percentages of screening group, previously screened, newly screened, and never screened. C-erb B2 expression is expressed as; Positive= c-erb B2 protein overexpression, Negative= no c-erb B2 protein overexpression.

Figure 36

C-erb B2 expression and cancer size (diameter in mm), expressed as percentage of cancer size. C-erb B2 expression is expressed as; Positive= c-erb B2 protein overexpression, Negative= no c-erb B2 protein overexpression.

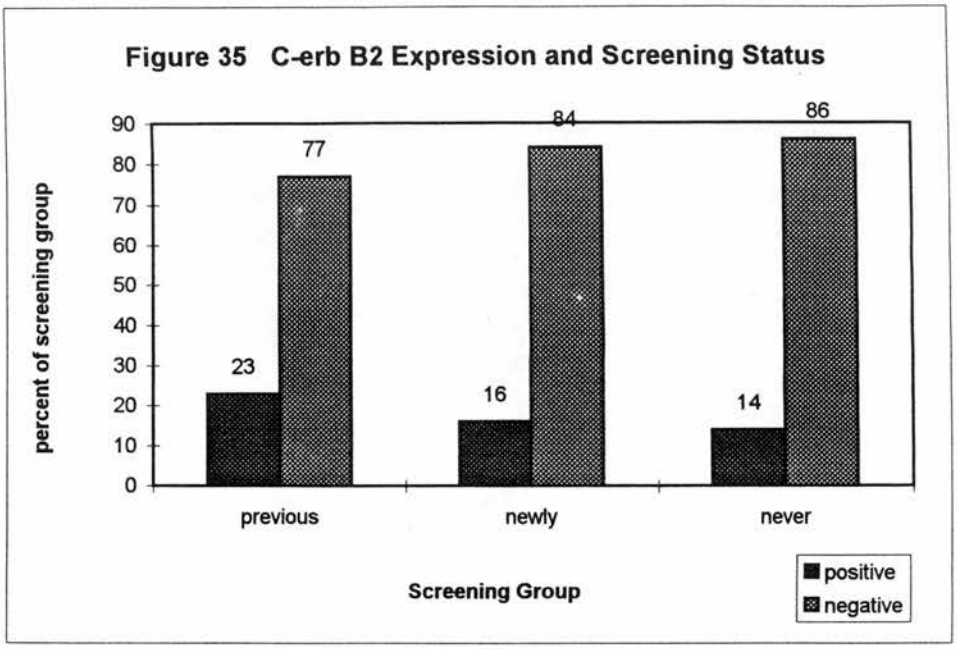


Figure 35 C-erb B2 Expression and Screening Status
percent of screening group
90
80
70
60
50
40
30
20
10
0
23
77
16
84
14
86
previous
newly
never
Screening Group
positive
negative

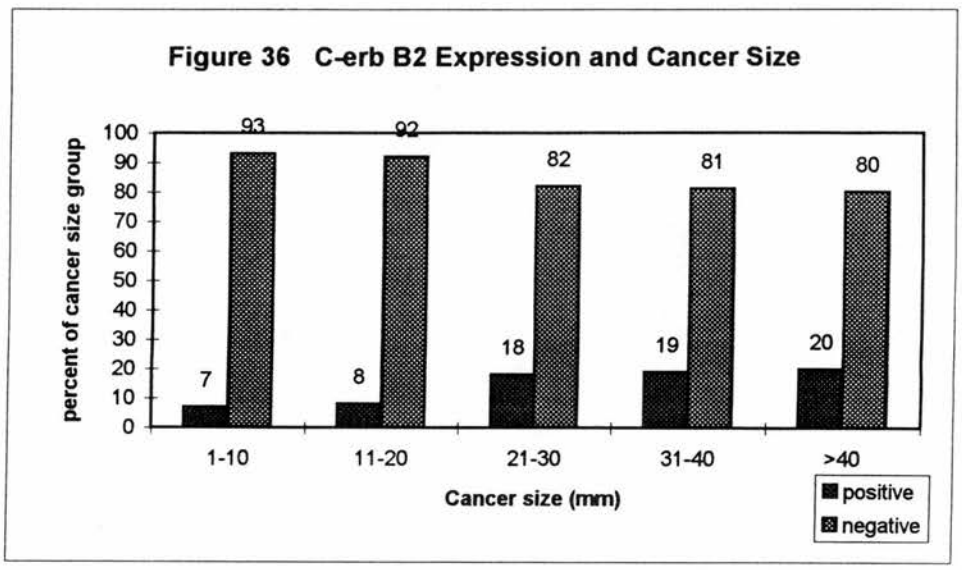


Figure 36 C-erb B2 Expression and Cancer Size
percent of cancer size group
100
90
80
70
60
50
40
30
20
10
0
7
93
8
92
18
82
19
81
20
80
1-10
11-20
21-30
31-40
>40
Cancer size (mm)
positive
negative

c-erb B2 expression in cancers <10mm was not due to the high frequency of special type cancers (commonly small and c-erbB2 negative). There was no correlation between lymph node status and c-erb B2 expression, $X^2= 0.04$, $p=0.5$.

The highest frequency of c-erb B2 overexpression was detected in *in situ* cancers (38%) with a lower percentage in invasive cancers (15%), described in Chapter 3, $X^2= 19.46$, $p<0.001$. In invasive cancers c-erb B2 overexpression was found predominantly in ductal carcinomas of no special type, with only one special type, a medullary, positive.

Significant associations were found between c-erb B2 overexpression and high cancer grade, $X^2= 10.17$, $p <0.025$ (Figure 37), and oestrogen receptor negative cancers, $X^2= 54.37$, $p<0.001$ (Figure 38). No grade I cancers showed overexpression, whereas grade II and grade III had 13% and 16% of overexpression respectively.

These results suggest that the frequency of c-erb B2 expression does not change with any of the chronological features of breast cancer, but is strongly associated with biological characteristics such as histopathological type and oestrogen receptor status. On multivariate analysis the significant association of expression with high grade was not independent of oestrogen status.

Figure 37

C-erb B2 expression and histopathological grade, expressed as percentage of cancer grade. C-erb B2 expression is expressed as Positive= c-erb B2 protein overexpression, Negative= no c-erb B2 protein overexpression. The frequency of cancers overexpressing c-erb B2 increases with increasing cancer grade.

Figure 38

C-erb B2 expression and oestrogen receptor concentration, expressed as percentage of oestrogen receptor (ER) group. Oestrogen receptors are categorised as <20fmol/mg protein, 20-200 fmol/mg protein, and >200fmol/mg protein. C-erb B2 expression is expressed as; Positive= c-erb B2 protein overexpression, Negative= no c-erb B2 protein overexpression. The frequency of c-erb B2 expression decreases with increasing oestrogen receptor concentration.

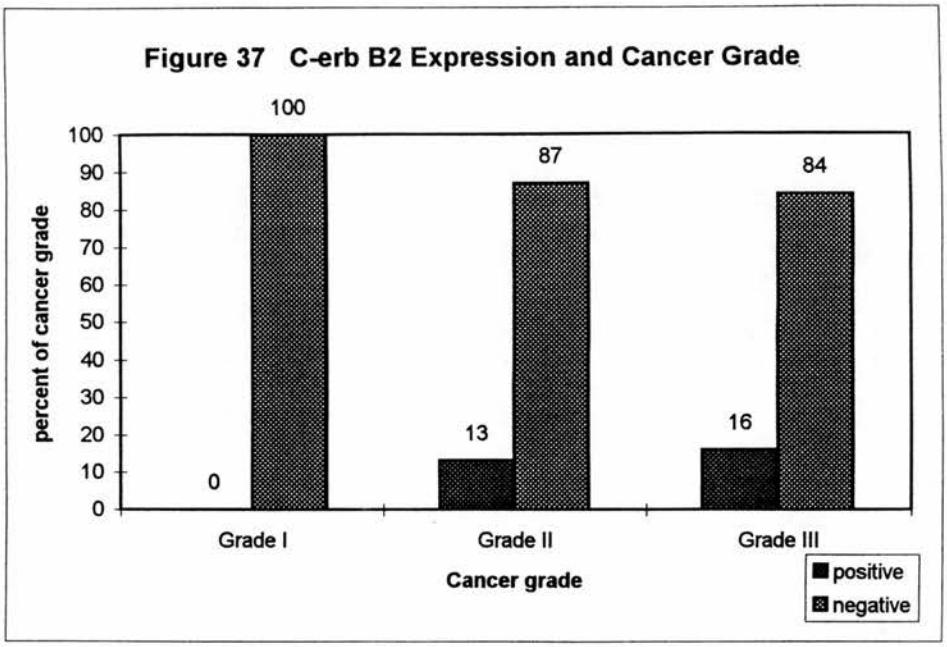


Figure 37 C-erb B2 Expression and Cancer Grade
percent of cancer grade
100
90
80
70
60
50
40
30
20
10
0
100
87
84
13
16
0
Grade I
Grade II
Grade III
Cancer grade
positive
negative

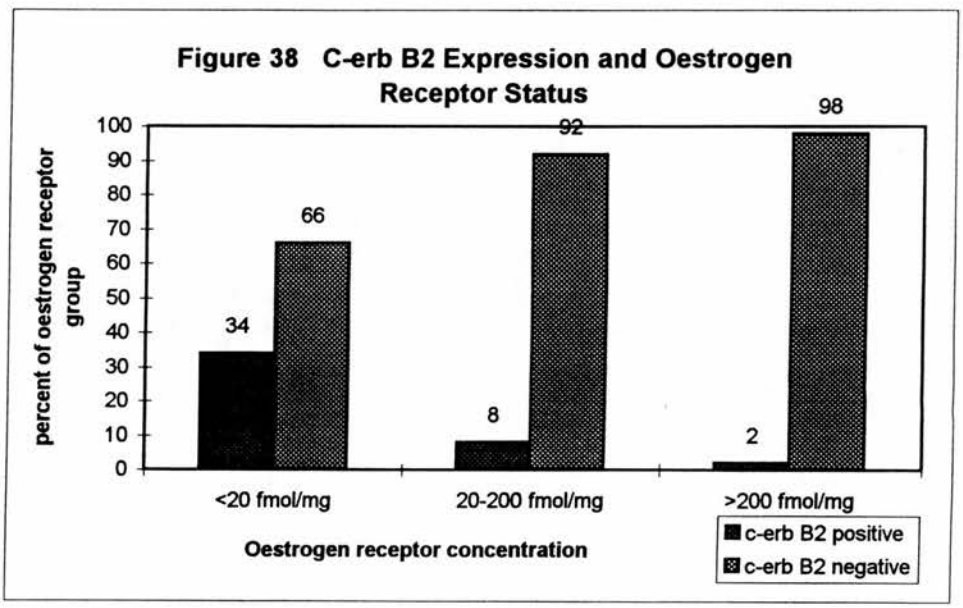


Figure 38 C-erb B2 Expression and Oestrogen Receptor Status
percent of oestrogen receptor group
100
90
80
70
60
50
40
30
20
10
0
98
92
66
34
8
2
<20 fmol/mg
20-200 fmol/mg
>200 fmol/mg
Oestrogen receptor concentration
c-erb B2 positive
c-erb B2 negative

4.3.5 C-erb B2 Amplification and Histopathological and Biological Characteristics of Breast Cancer.

In univariate analysis, c-erb B2 amplification was found to vary with oestrogen receptor status and showed minor differences in distribution with cancer type, DNA ploidy and cancer size. The frequency of c-erb B2 amplification was independent of lymph node status, screening status and cancer grade. C-erb B2 gene amplifications were present in 55% of the cancers in the never screened group, and in 52% and 61% of previous and newly screened respectively, Figure 39, these differences in frequency were not significant, X^2= 1.03, p=0.50.

There was no significant correlation between the presence of gene amplification and cancer size, X^2= 6.97, p<0.10. Similar percentages of cancers with c-erb B2 amplification were detected in four groups 10mm in size ranging from 11 to >40 mm (46% to 58%). Cancers of 10mm or less in diameter appeared more likely to be amplified (71%, Figure 40), but this was not statistically significant. Frequencies of c-erb B2 amplification for lymph node positive (54)% and negative cancers (55%) were similar, X^2= 0.03, p=0.5.

C-erb B2 gene amplification was present 55% of invasive cancers and 65% of *in situ* cancers, see Chapter 3. Amplification was present in lobular (80%), tubular (58%), medullary (67%), and DCI no special type (52%). C-erb B2 did not correlate with cancer grade, and was present in 49%, 54% and 45% of grades I, II, and III respectively, X^2=1.03, p>0.50. A significant correlation between ER concentration and c-erb B2 amplification was observed, Figure 41, X^2 = 10.47, p = < 0.01. On multivariate analysis this association was not independent of c-erb B2 expression.

Figure 39

C-erb B2 gene amplification and screening status, expressed as a percentage of screening group, previously screened, newly screened, and never screened. C-erb B2 amplified cancers = dPCR ratio values of 2 or above, non amplified cancers = dPCR ratio values <2.

Figure 40

C-erb B2 gene amplification and cancer size (diameter in mm), expressed as percentage of size group. C-erb B2 amplified cancers = dPCR ratio values of 2 or above, non amplified cancers = dPCR ratio values <2. Cancer sizes have been grouped in 10mm categories.

Figure 39 C-erb B2 Gene Amplification and Screening Status

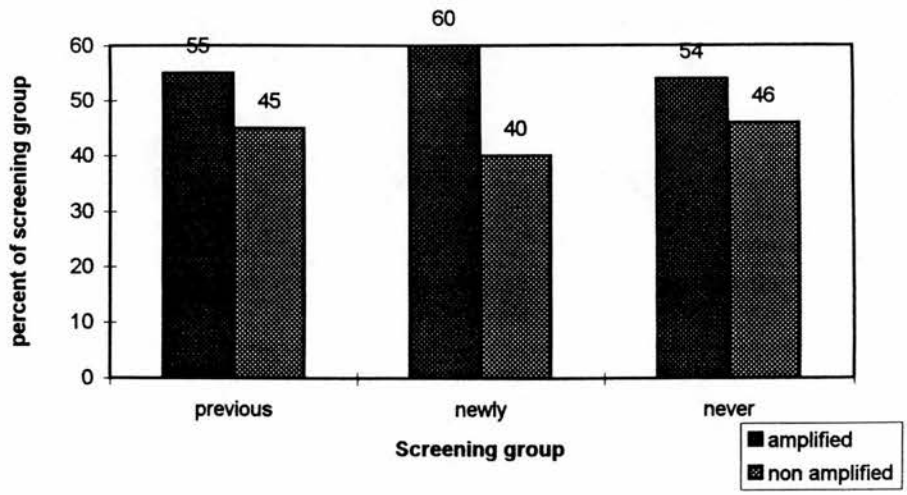


Figure 40 C-erb B2 Gene Amplification and Cancer Size

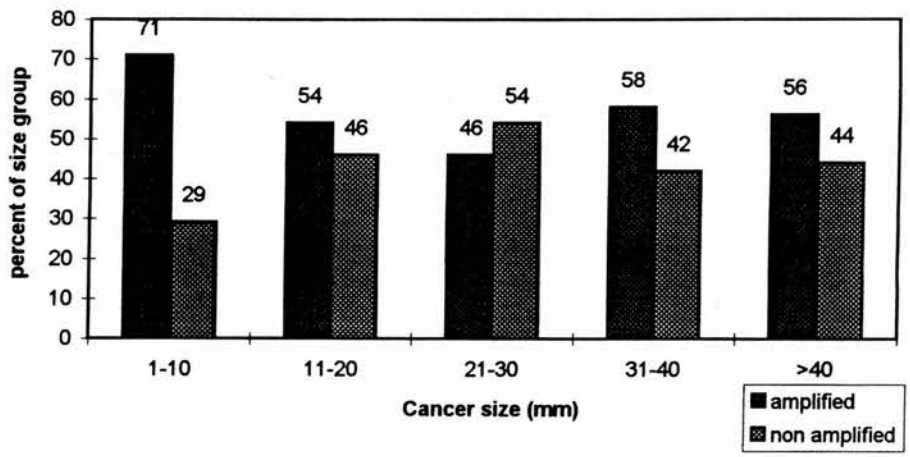
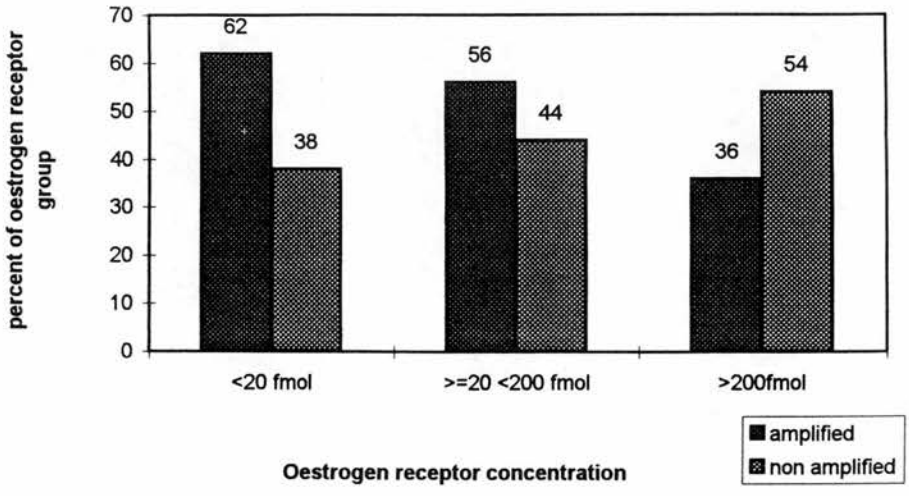


Figure 41

C-erb B2 gene amplification and oestrogen receptor status, expressed as percentage of oestrogen status group, <20fmol/mg protein, 20-200 fmol/mg protein, and >200fmol/mg protein.. C-erb B2 amplified cancers = dPCR ratio values of 2 or above, non amplified cancers = dPCR ratio values <2.

Figure 41 C-erb B2 Gene Amplification and Oestrogen Receptor Status



C-erb B2 gene amplification was most frequent in diploid cancers (60%) with frequencies of 52% and 51% in aneuploid and tetraploid cancers respectively, these differences were not significant, $X= 3.06$, $p = <0.50 >0.10$.

These results suggest that the frequency of c-erb B2 gene amplification does not change with chronological features and does not appear to be associated with biological cancer features investigated in this study.

4.4 DISCUSSION

Data presented within this chapter provide valuable insights into the biological and chronological features of breast cancers which formed part of a breast screening program. The relationships between various features is important in the identification of markers of cancer progression, and the evaluation of molecular events. As yet, no invariant markers have been identified. This is probably due to the considerable heterogeneity of breast cancer disease and to the fact that examinations of cancer tissues usually represents only a single "time frame". As screen detected cancers may represent a different phase in cancer chronology from symptomatic cancers, I attempted to identify those cancer features which differed in distribution between these sets and how these could be related to cancer progression. The contribution of c-erb B2 disregulation to breast cancer progression was assessed using this "framework" of characteristics.

The importance of comparing cancers which have been detected mammographically with those detected symptomatically cannot be overstated, as this is the only means at present to obtain breast cancers at different stages of chronology. Early lesions of the breast are not well characterised (Cardiff 1988), and the critical molecular events in oncogenesis have not been identified or related to histopathology or cancer features assessed in this chapter. Current theories of breast cancer progression suggest that cancers arise following the transition of normal breast cells to hyperplasia, to *in situ*, to invasive, to metastatic carcinoma (Cardiff 1988). Other features, such as cancer size, cancer grade, lymph node status, oestrogen receptor status, and DNA ploidy are also likely to change with this cancer progression (Tubiana and Kosceilny 1991), each being an indicator of tissue differentiation, metastasis or DNA damage. However the exact relationship of these features to

progression is ill-defined. In addition, by the time an *in situ* or invasive cancer is clinically detected it is likely to contain a number of molecular disregulations, not all of which may be of direct relevance to cancer progression.

4.4.1 Screening Status and Cancer Characteristics.

Cancers detected by mammographic screening are usually non-symptomatic, and should represent a chronologically earlier population of cancers than those presenting symptomatically (Miller 1992), although this may represent only a relatively short period in relation to the complete history of the cancer. In agreement with other studies (Anderson et al 1991), the mammographically screened cancer sets in this study contained a higher proportion of small cancers, and *in situ* and special type cancers. Differences in the detection of cancer types may not necessarily indicate different stages of progression, however some current theories suggest that special type cancers could progress to invasive ductal carcinoma of no special type (Cardiff 1988, Tubiana and Koscielny 1991). The occasional co-existence of different morphological forms within the same cancer would support this suggestion, however evidence of true progression remains circumstantial. It is likely that the only feature, apart from cancer type, which changes significantly over this "window" of cancer progression is cancer size. Similarly lymph node involvement was strongly correlated with larger cancer on multivariate analysis.

Few clear differences in cancer grade, DNA ploidy, lymph node status and oestrogen receptor status were observed between the sets. This may be partly due to the nature of the newly screened set. These cancers were detected during the prevalence screen

and included some cancers at a relatively advanced, but sub-symptomatic stage, as illustrated by the presence of some cancers greater than 40mm in size. The previously screened set was too small for full analysis of breast cancer characteristics, however a small number of cancers within this group had an unexpected combination of features. The previously screened set had a higher proportion of grade III cancers than either of the other sets, and these cancers were largely negative for oestrogen receptors and lymph node involvement. This suggests that they are rapidly growing , poorly differentiated cancers, features not normally associated with an early stage of progression. Unfortunately the exact time interval between a clear mammographic screen and detection of the cancer was not available. The newly screened and previously screened groups therefore appear to represent slightly different sets of breast cancer.

Overall these results indicate that screening does detect some cancers at an earlier stage of progression, however there remains considerable heterogeneity within each set. Therefore, other potential markers of cancer progression remain important.

4.4.2 Histopathological and Biochemical Features of Breast Cancer and Cancer Progression.

Cancer features can be classified according to "good" or "bad" characteristics indicated by the data analysed in this study, see Table 20, and a simplistic hypothetical "framework" of features relating to cancer progression suggested. Early stages of cancer progression may be associated with low grade, oestrogen receptor positivity, *in situ* or special type cancers, small size and lymph node negativity. Whereas late stages of cancer progression may be associated with high grade, large

Table 20. A simplistic model of cancer characteristics and their association with cancer aggression.

Table 20

Cancer features	***good***				***bad***
Cancer Size	small	→			large
Lymph node status	negative	→			positive
Cancer grade	I	→	II	→	III
Oestrogen receptor status	positive	→			negative
DNA ploidy	diploid	→			aneuploid

size, oestrogen receptor negativity, ductal carcinomas of no special type and lymph node positivity.

Cancer features have been positioned in Table 20 according to their relative associations found in this study. Assessment of screening groups with size and lymph node status established them as chronological factors (section 4.4.1). Both histopathological grade and DNA ploidy status were significantly correlated with cancer size, suggesting breast cancer cells become more poorly differentiated and show more disturbance to DNA ploidy with cancer progression, indicated by cancer size. An association between cancer grade and progression has been observed by others (Tubiana and Koscielny 1991), although it is unclear how many cancers will remain at the same grade throughout their "life". The speed at which transition occurs between grades could be very variable (Tubiana and Koscielny 1991), a conclusion supported in this study by the finding of some large cancers of low grade (Table 31). Changes to DNA ploidy are also thought to increase with cancer progression (Bacus et al 1990). In this study small cancers were clearly more frequently diploid, but aneuploid and tetraploid cancers did not increase in frequency in cancers over 20mm in size, suggesting that the relationship between DNA ploidy and progression indicated by cancer size may only apply over a relatively short size range.

The percentage of lymph node positive cancers increased with cancer size, indicating that the larger, growing cancers have more cells which are able to grow in the nodes. However, positive lymph nodes were not strongly associated with other markers of aggression such as changes in DNA ploidy, cancer grade, oestrogen receptor negativity, and did not vary greatly with screening status. A model proposed by

Tubiana and Koscielny (1991) suggested that there was a continual progression from an initial lack of involvement of lymph nodes to the involvement of one or two nodes as the primary cancer volume increases. While the number of involved nodes may be important in an assessment of cancer prognosis, lymph node sampling and assessment was not extensive or complete in the surgical operations where my samples originated (usually three of potentially >20 nodes).

Whilst oestrogen receptor concentration was not significantly associated with many indicators of progression, a strong correlation between low oestrogen receptor concentration and high cancer grade would indicate that loss of oestrogen receptor is associated with "late" stage disease. This has been confirmed in other studies, which also found correlation with aggressive breast cancer (Schroeter et al 1992, Slamon et al 1989, Tsuda et al 1989). However oestrogen receptor expression may be controlled by several mechanisms (Piva et al 1988, Piva et al 1993, Kameko et al 1993) and should be regarded as a biological marker rather than a chronological marker of progression.

4.4.3 Overexpression of c-erb B2 and Cancer Progression.

The high frequency of disregulation of c-erb B2 suggests that it may play a role in the aetiology of breast cancer. However, it is not clear at what point(s) of progression the disregulation of c- erb B2 may occur, or what its contribution to the disregulated phenotype may be. A model of cancer progression involving c-erb B2 in breast cancer has been proposed by Allred et al (1992). They proposed that c- erb B2 expression is an early or initiating event in some cancers, particularly comedo type *in situ* cancers, only a proportion of which progress to invasive carcinomas, or that c-erb B2 expression is reduced on transition from *in situ* to invasive carcinoma. In

addition, they suggest that many invasive cancers progress to invasive ("de novo") without a significant *in situ* stage and without c-erb B2 expression. This model is based on histopathological features of breast cancer, and does not fully discuss the potential effect of factors which may alter the expression of the gene.

In this study of c-erb B2 expression was associated with both "early" and "late" stage cancer characteristics. C-erb B2 overexpression has not been detected in putative pre-neoplastic lesions (Allred et al 1992, Gusterson et al 1988, De Potter et al 1989) but appears to be associated with *in situ* or invasive types of cancer. In agreement with several other studies (Allred et al 1992, Barnes et al 1992, Gusterson et al 1988, De Potter et al 1989), I found that c-erb B2 overexpression was significantly associated with certain cancer types, with the highest frequency being detected in *in situ* cancers, see Chapter 3 Table 12. This may suggest that c-erb B2 expression is a marker of the transition between these stages of development in some breast cancers, providing that the cancer type changes with progression. Because the frequency of c-erb B2 overexpression did not appear to vary with screening status, lymph node status, DNA ploidy disturbance, or cancer size, this suggests that c-erb B2 expression can be an early, stable event.

In apparent contradiction to the results discussed above, c-erb B2 expression was also significantly associated with high cancer grade and oestrogen receptor negativity, both potential markers of late stage disease. This has been observed in other studies (Russell and Hung 1992, Todd et al 1992), and may indicate a functional relationship between c-erb B2 and oestrogen receptor. Oestrogen receptor functions as a nuclear receptor involved in transcriptional regulation of target genes (King 1992), and may have the potential to down regulate the

expression of c-erb B2. This has been shown to occur *in vitro*, where c-erb B2 expression in breast cancer cell lines can be down-regulated by oestrogen receptor complexed with oestradiol (Russell and Hung 1992, Todd et al 1992). Results here indicate that this also occurs *in vivo*, indicating that c-erb B2 expression is not related to late stage disease but has a biological association with oestrogen receptor.

These associations indicate that expression of c-erb B2 may be an early event, however its biological effect on cancer progression remains unclear.

4.4.4 C-erb B2 gene Amplification and Cancer Progression.

C-erb B2 expression is closely associated with c-erb B2 gene amplification, see Chapter 3, but it remains unclear whether these events are separate disregulations or causally related. A high percentage of cancers in this study had c-erb B 2 gene amplification in the absence of overexpression, but as discussed above, c-erb B2 expression can be regulated by other factors, therefore the relationship between these events and to cancer progression is difficult to establish. C-erb B2 gene amplification has been proposed in some studies as a late event (Kraus et al 1987), but this was based on the observation that expression was more frequent especially in *in situ* cancers.

In contrast, results in this study suggest that c-erb B2 amplification may be an "early" genetic event that is maintained during cancer progression and does not greatly change in frequency between screening groups. Its common presence in all cancer types, including special types, which are almost universally negative for c-erb B2

overexpression, suggests that it may be associated with a fundamental change in the normal breast epithelial cell. Indeed c-erb B2 gene amplification is more frequent in *in situ* cancers than invasive cancers, see Chapter 3. C-erb B2 gene amplification was independent of cancer size, lymph node status, cancer grade, and disturbance to DNA ploidy. A weak negative correlation with oestrogen receptor was found, but this was not independent of c-erb B2 expression. This lack of association with "late" stage markers also supports an early role for gene amplification. This finding contrasts with other studies of amplified genes, where amplification of EGFR and n-myc were associated with late stage (III and IV) cancer (Brodeur et al 1984, Ekstrand et al 1991, Ranzani et al 1990).

The mechanisms of c-erb B2 gene amplification are poorly understood, but are unlikely to be due to purely random chromosomal disruption. Changes in DNA ploidy generally indicate progressive cellular disregulation, cancer cells are thought to become tetraploid during cancer development, then lose extra chromosomes in a random fashion over time to become aneuploid (Bacus et al 1990). In this study there was no association of c-erb B2 gene amplification with increasing DNA ploidy disturbances, this concurs with a large study (Borg et al 1991), and suggests that gene amplifications are due to stable changes in chromosome structure, not detectable by DNA ploidy analysis.

4.4.5 Conclusions

Cancer size and lymph node status have been identified as chronological markers of breast cancer and a hypothetical framework of cancer characteristics in relation to cancer progression is proposed. While it is clear that the role of c-erb B2 in the progression of breast cancer is complex, disregulation of c-erb B2 does appear in

relatively early cancers. Amplification does not appear to increase or decrease with features of late stage disease, and may suggest that it is a relatively stable event. C-erb B2 overexpression also occurs in "early" cancers, but expression may be temporal and depend on transcriptional control mechanisms. However not all cancers have detectable c-erb B2 disregulation, therefore alternative pathways of cancer progression which do not involve c-erb B2 disregulation exist.

CHAPTER 5

Possible Allelic Imbalance of c-erb B2 in Breast Cancer.

5.1 INTRODUCTION

The high frequency of c-erb B2 disregulation in breast cancer, especially gene amplification, suggests that it represents a specific event, selected for in the process of breast carcinogenesis. The precise chromosomal location and extent of c-erb B2 amplification in cancer tissues is still unclear. Amplifications of greater than 40 copies of c-erb B2 have been reported (Ali et al 1988, Berns et al 1992, Tavassoli et al 1989) but most breast cancers harbour less than 10 extra copies of c-erb B2 (Chapter 3, Borg et al 1991). A biological selection pressure for c-erb B2 amplification may exist in breast cancer, but very high gene copy number may also indicate an amplification mechanism which is "jammed on". *In situ* hybridisation studies have suggested that c-erb B2 amplifications are present at more than one chromosomal location and that the copy number is variable between cells (Smith et al 1993), but it is not known whether c-erb B2 amplifications are present on chromosome 17q, in homogeneously stained regions within other chromosomes, or as extra-chromosomal DNA forming double minutes.

It is not known whether one or both alleles are involved in c- erb B2 amplification and whether amplification is a random process, equally as likely to affect each allele. Investigation of possible associations between gene amplification and genotype, and analysis of relative allele intensity may provide some clues to processes involved in gene amplification. Amplifications may arise from either disregulated DNA replication mechanisms or disregulated gene transcriptional mechanisms (Stark et al 1989), see Chapter 1. Gene overexpression prior to gene amplification has been proposed as a key factor in gene amplification of the multiple drug resistance gene 1 in cell lines (Shen et al 1988) and of c-erb B2 in breast cancers (Kraus et al 1987). This may involve disregulation of gene expression, in which increased or altered

transcription may lead to a malfunction of normal DNA replication resulting in re-replication of newly replicated DNA in S phase of the cell cycle (Stark 1989). This process could involve gene promoters, or suppressers, which may act upon both alleles of c-erb B2, assuming that the transcriptional control regions of each allele are the same. Uneven recombination followed by amplification could also result in amplification of both alleles in one cell lineage, accompanied by complete loss in another. Alternatively, preferential amplification of one allele could suggest a genetic predisposition associated with that allele. Predisposition could result from a functional difference associated with a polymorphism or a genetic linkage to another relevant DNA sequence. Purely random amplification mechanisms would effect either or both alleles.

Several oncogenes have shown allelic imbalance in various cancer types, such as c-Ha-ras in breast cancer and L-myc in bone and soft tissue sarcomas (Honda et al 1988, Kato et al 1990). It has been suggested that a higher frequency of rare alleles in cancers than in the normal population may indicate a predisposition to carcinogenesis associated with that allele (Honda et al 1988, Kato et al 1990). However, this remains only a possibility and has not been experimentally confirmed. Genes which show allele imbalance in breast cancer are infrequently amplified, therefore any potential relationships have not been identified. Other oncogenes, such as c-myc (Riou et al 1984, Riou et al 1987), which can be amplified in breast cancer have not been investigated for allele specific amplification.

A restriction fragment polymorphism of c-erb B2 permits the analysis of the genotype and of the relative intensities of c-erb B2 alleles in breast cancer DNA. The polymorphism is present in the general population, and has a polymorphism

information content of 0.3, that is 30% of the population will be heterozygous at this locus. The polymorphic site is formed by a single base pair insertion within intron 3 of the gene which creates an additional *MboI* restriction site, and is detected on restriction digestion of a 1.1kb PCR amplified DNA sequence spanning the polymorphic site (Hall et al 1990a).

This study aimed to;

1) Determine the allele frequencies and allelotypes for the c- erb B2 polymorphism in c-erb B2 amplified and non-amplified breast cancers and in control DNAs.

2) Assess relative band intensities in heterozygous patients and controls to identify whether one or two alleles have been amplified.

5.2 MATERIALS AND METHODS

5.2.1 Tissues

Breast cancer tissues were obtained at routine surgical operations, as described in Chapter 3. Each tissue was snap frozen and stored at -70°C. Samples were restricted to cancers of sufficient size to provide tissue for DNA extraction. These varied from 7mm to 80mm in diameter. *In situ* cancers were not measured. Most of the cancers were invasive of no special type (DCI NST), 5 were *in situ* carcinomas, 4 were invasive lobular, 2 were invasive tubular and one was mucinous. Control tissue was obtained from non involved colon tissue from colon cancer patients. Tissue DNA was prepared by standard methods (Sambrook et al 1988) and resuspended to a final concentration of 1mg/ml. Histopathological characterisation of the tissues was performed on fixed tissue taken adjacent to the frozen portion. As described in Chapter 3, the proportion of "contamination" by normal stromal cells was assessed subjectively in these fixed tissues.

Any c-erb B2 amplification was determined by differential polymerase chain reaction on DNA from fixed tissues and DNA ploidy changes were assessed by flow cytometry. Both methods are described in Chapter 3.

5.2.2 Allele Analysis

For each specimen a 1.1kb fragment of the c-erb B2 gene was amplified using specific primers spanning the polymorphic site: sense 5'CTG GAA TGG GAA GCA 3' and antisense 5'GCC AGC AAA GAA ATC TTA GAC GT 3' (Hall et al 1990a), with 200ng of template DNA, 250pmol of each primer, 200mM dNTPs (Pharmacia), 1x reaction buffer (Bioline, UK), and 0.5 unit Taq polymerase (Bioline, UK) in each

tube for a PCR reaction. Cycling was performed on a Hybaid Omnigene thermal cycler incorporating parameters of one cycle of 94°C for 5 mins, 55°C for 1 min, 72°C for 1 min 30 sec, followed by 30 cycles of 94°C for 1 min, 55°C for 1min, 72°C for 1 min 30 sec, and one cycle of 94°C for one min, 55°C for 1 min and 72°C for 5 mins. Each PCR product was digested twice with 3 units of restriction enzyme *Pvu II* (Sigma, UK) at 37°C for 3 hours. Buffer concentrations of Tris HCl and NaCl were altered to give a final concentration of 50mM Tris HCl pH 7.9 and 100mM NaCl. Samples were then digested twice with 3 units of *Mbo I* (Sigma) at 37°C for a further 3 hours, to digest the PCR products into the allelic bands of 520 or 500 bp. The *Pvu II* restriction was necessary to pre-digest a constant band of 550 bp, which would appear with *Mbo I* digestion alone, and would confuse identification of the *Mbo I* allelic bands.

Restriction fragments were separated by size through a 1.8% Metaphor agarose gel (FMC products, UK) for 2 hours at 125V, stained by ethidium bromide, visualised in ultra violet light, and photographed. Each specimen was tested in duplicate experiments. A molecular weight marker (1.1kb ladder) was run in an adjacent gel track to each sample. Allelotypes and relative band intensity were assessed visually, and scored independently by myself and another scientist (Dr V.J. Bubb, Dept. Pathology, Edinburgh).

5.2.3 Population Analysis

Allele frequencies for each group were calculated and genotype distribution for amplified, non amplified and controls were tested for goodness of fit with the Hardy-Weinberg equilibrium. The Hardy-Weinberg theorem is used to calculate the frequencies of homozygotes and heterozygotes within a population. The theorem is based on the principle that in the absence of migration, mutation, and selection, gene

frequencies remain constant in a large population. Expected genotype distribution is calculated from the observed allele frequencies using the equation p2 + 2pq + q2 =1, where p and q represent the frequency of each allele. Deviations from this equilibrium are calculated using a chi-squared test on observed and expected frequencies. Two by two contingency tables and chi-squared statistics were used to assess differences in allele frequencies between groups.

5.3 RESULTS

5.3.1 Allele analysis and Frequencies.

A 1.1kb fragment of c-erb B2 was PCR amplified and digested from 70 breast cancers and 34 controls. Digestion with *Mbo I* and *Pvu II* resulted in one or two allelic bands of 520bp (A1) and 500bp (A2), and two constant bands of 330bp and 220bp, as illustrated in Figure 42. All digests with *Mbo I* and *Pvu II* were complete, with no evidence of residual PCR product after gel electrophoresis of digested product.

The distribution of genotypes for amplified, non-amplified and control tissues are shown in Table 21. Allele frequencies determined from the distribution of genotypes within each group were not significantly different between "total" cancers and controls. Nor were there significant differences in allele frequencies between amplified and non amplified cancers, Table 22, X2= 2.01 , p= 0.2-0.10.

5.3.3 Genotype Distribution.

Chi-squared analysis of observed genotype distribution for each group did not deviate significantly from expected frequencies, Table 21. Further analysis of genotype distribution in the amplified cancer group using the allele frequencies of the normal and non amplified groups, Tables 23 A and B, demonstrated a deviation from the Hardy Weinberg equilibrium. These differences were statistically significant when non amplified allele frequencies were used in the equation, X2 = 9.12, p=<0.01, and when control alllele frequencies were used in the equation, X2= 5.99, p= <0.05 ->0.01

Figure 42

Photograph illustrating a restriction fragment length polymorphism contained within intron 3 of c-erb B2. Breast cancer DNA in tracks 1-6 represent an *Mbo I* and *Pvu II* restriction digest of a 1.1kb PCR amplified sequence of c-erb B2. Allelic bands are indicated at 520bp (A1) and 500bp (A2), constant bands of 330bp (C1) and 220bp (C2) are indicated. Track 1 shows a homozygous A2 DNA, track 2 shows a homozygous A1 DNA, and tracks 3, 4, 5, 6 are show heterozygous DNA. Allelic bands in tracks 3, 4, and 6 show uneven band intensity.

Figure 42

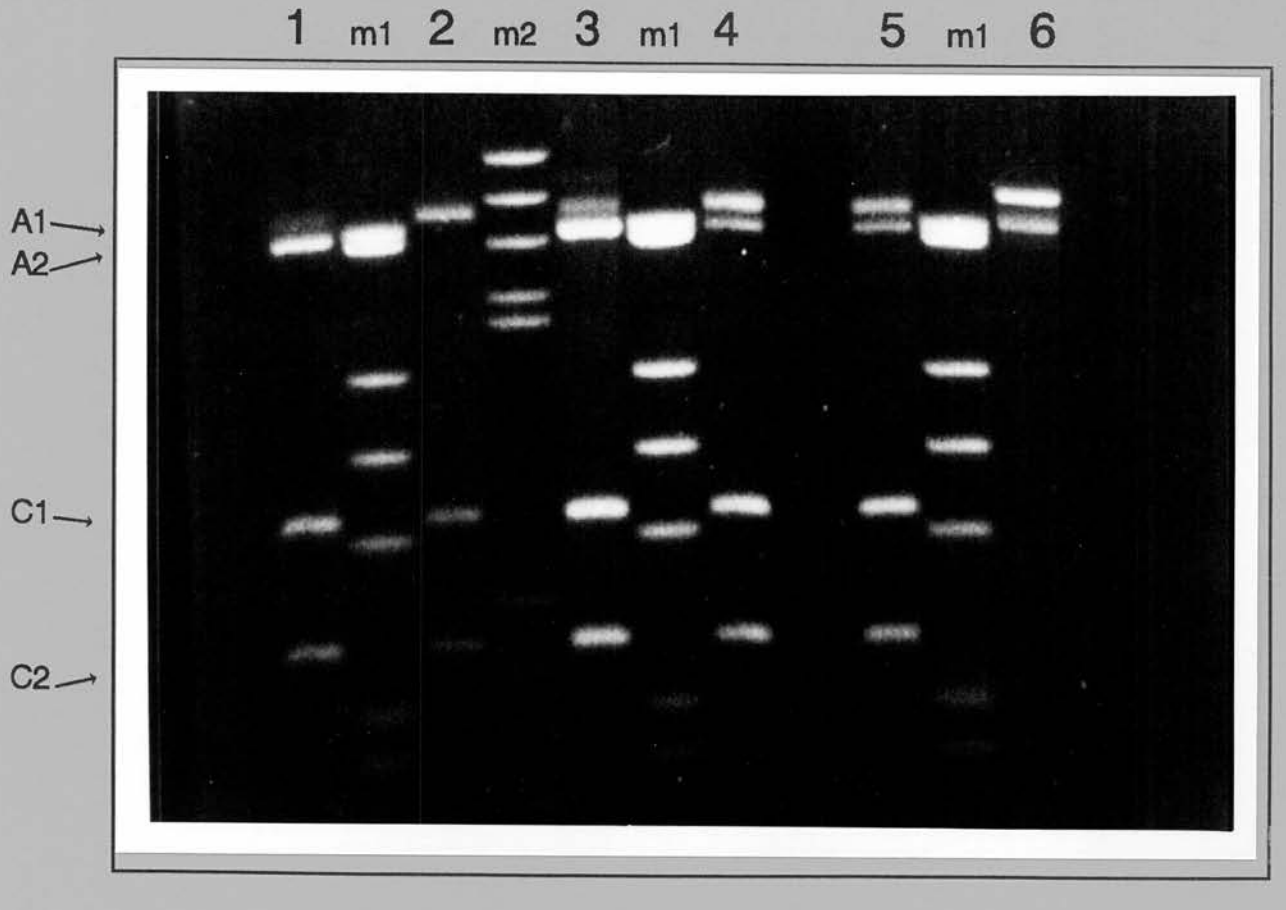


Table 21

Genotype distribution of breast cancers groups with (Amplified) and without (Non-Amp) c-erb B2 gene amplification, and a normal control group (Control). Allele frequencies were determined from observed genotypes (O) for each group (p = A1, q = A2), and the expected genotype frequencies (E) calculated according to the Hardy-Weinberg equilibrium (p2+2pq+q2=1). Chi-squared statistics were performed for each group and p values given

			Genotypes			Allele Frequencies	
Group	**n**	**O/E**	**A1/A1**	**A1/A2**	**A2/A2**	**p**	**q**
Amplified	**34**	**O**	**15**	**12**	**7**	**0.62**	**0.38**
		E	**13**	**16**	**5**		
			X2=2.11, p= >0.2 <0.5				
Non-amp	**38**	**O**	**21**	**13**	**4**	**0.72**	**0.28**
		E	**20**	**15**	**3**		
			X2=0.64, = >0.2 <0.5				
Control	**37**	**O**	**20**	**11**	**6**	**0.69**	**0.31**
		E	**18**	**16**	**4**		
			X2=2.78, p=0.10				

Table 22
Observed allele frequencies (A1 and A2) for c-erb B2 amplified and non amplified breast cancers. Allele frequencies were compared in a two by two contingency table. Chi-squared statistics showed that allele frequencies were not significantly different between c-erb B2 amplified and non amplified cancers.

	Allele A1 (p)	Allele A2 (q)	total
Amplified	**42**	**26**	**68**
Non-amplified	**55**	**21**	**76**
total	**97**	**47**	**144**

X2= 2.01, p= 0.1-0.2

Table 23
Distribution of c-erb B2 genotypes in c-erb B2 amplified cancers. Expected values according to the Hardy-Weinberg equilibrium were calculated using allele frequencies of A. = nonamplified cancers, and B = normal controls. Chi-squared analysis was performed on observed and expected genotype frequencies, and p values given.

A

	Genotypes			
	A1/A1	A1/A2	A2/A2	X2
observed	**15**	**12**	**7**	**9.12**
expected	**17**	**14**	**3**	**p=<.01**

B

	Genotypes			
	A1/A1	A1/A2	A2/A2	X2
observed	**15**	**12**	**7**	**5.99**
expected	**16**	**15**	**3**	**p=.05-.01**

Distribution of genotypes with changes of DNA ploidy are shown in Table 24. The c-erb B2 genotype distribution in cancers which were diploid and tetraploid showed no deviation from Hardy-Weinberg equilibrium, however a reduced frequency of heterozygotes was observed for aneuploid cancers which approached significance. Study numbers were too small to subdivide each ploidy group by amplification status.

5.3.4 Allele amplification.

Heterozygotes were assessed for allele band intensity, and scored as even intensity (e), more intense allele 1 (A1), or more intense allele 2 (A2), Table 25. All control heterozygotes had even intensity bands. Thirty one percent of heterozygous non-amplified cancers showed uneven band intensity, equally distributed between A1 and A2. However, 58% of heterozygous amplified cancers showed uneven band intensity, with 5 of 7 of the most intense bands being A2.

Case numbers were too small for any clear assessment of possible associations between genotype distribution and clinical characteristics, such as screening status, cancer type, cancer grade, lymph node involvement and oestrogen receptor status. Eleven of the study cases overexpressed c-erb B2 and all but one of these had gene amplification. Deviation of genotype distribution from the Hardy-Weinberg equilibrium was observed in c-erb B2 overexpressing cancers, and approached significance, $X2 = 3.6$, $p = 0.05$. A larger study is necessary to fully evaluate this finding.

Table 24

Distribution of c-erb B2 genotypes with DNA ploidy status, diploid, tetraploid and aneuploid. Allele frequencies were calculated for each group (p and q), and expected values determined according to the Hardy-Weinberg equilibrium and are given in parenthesis. Chi squared statistics were performed on observed and expected values, and p values given.

	Genotype					
DNA Ploidy	**A1/A1**	**A1/A2**	**A2/A2**	**A1 (p)**	**A2 (q)**	**X2**
Diploid	**9 (10)**	**11 (10)**	**2 (2)**	**0.66**	**0.34**	**0.2 ns**
Tetraploid	**10 (9)**	**5 (7)**	**2 (1)**	**0.73**	**0.26**	**1.68 ns**
Aneuploid	**12 (10)**	**5 (10)**	**5 (2)**	**0.66**	**0.34**	**7.4 p=<0.05**

Table 25

Allele analysis of c-erb B2 polymorphic bands in heterozygous breast cancers and controls. The relative intensity of allelic bands on agarose gels was assessed visually, and recorded as A1 or A2 where the respective allele was most intense, or E, where heterozygous bands were of equal intensity. Only breast cancer cases showed uneven band intensities.

Group	**Heterozygote band intensity**		
	A1	**E**	**A2**
Amplified	**2**	**5**	**5**
Not amplified	**2**	**9**	**2**
Control	**0**	**11**	**0**

5.4 DISCUSSION

Analysis of frequency and distribution of c-erb B2 has identified differences in genotype distribution between cancers with and without c-erb B2 gene amplification. Allele A2, the 500 bp *Mbo 1* allelic band, may occur more frequently in c-erb B2 amplified cancers, than in non-amplified cancers or normal colon tissue. Where cancer tissue DNA was heterozygous at the c-erb B2 locus in c-erb B2 amplified cancers, allele A2 formed a disproportionately high percentage of the alleles amplified. These findings may indicate a bias in allele amplification.

The allele frequency and genotype distribution of the *Mbo 1* site c-erb B2 polymorphism for the control population in this study were both similar to those published for a normal American population (Hall et al 1990a). Allele frequencies can differ between populations physically isolated from one another and between different ethnic groups (Chakraborty 1990, Devlin et al 1993) and genotypes can deviate from the Hardy-Weinberg equilibrium (Sullivan et al 1992), complicating the interpretation of genotype distribution. However, it appears that allele frequencies at the c-erb B2 locus are stable, at least for those with European ancestry, and that the genotype distribution is not subject to selection pressure within the normal population.

In this study, allele frequencies were similar for control and cancer populations. However when cancers were analysed in c- erb B2 amplified and non amplified groups a difference in allele frequency was apparent, but did not reach significance. Analysis of a larger study set may resolve this trend, which indicated that allele A2 may be more frequent in c-erb B2 amplified cancers. Disturbances to allele frequency have been reported in cancer populations, including oncogenes L-myc and

c-Ha-ras, and may indicate that there is an association of specific alleles with initiation or progression of cancer (Champeme et al 1992, Kato et al 1990, Kawashima et al 1992, Lidereau et al 1986). Possible reasons for an excess of rare alleles in a population include population "bottlenecks", the presence of slightly deleterious mutations or hidden sub-divisions within populations (Chakraborty 1990). Hidden sub-divisions may become apparent when the rare alleles are associated with factors which become phenotypic, such as the development of cancer.

Selection pressure may also be apparent in the distribution of genotypes within a population. The distribution of genotype in the amplified cancer group deviated significantly from those expected according to the Hardy-Weinberg equilibrium, suggesting that a selection pressure may apply to this cancer group. It is possible that the selection pressure may be linked to gene amplification, as other cancer features are heterogeneous within this group. C-erb B2 amplified cancers showed an increase in A2 homozygotes and a reduction in A1/A2 heterozygotes, suggesting a potential bias associated with amplification. This bias for c-erb B2 genotype distributions may extend to cancers which overexpressed c-erb B2 protein, but case numbers were not sufficiently large for satisfactory analysis.

Deviations from the expected genotype frequencies have been reported in both normal populations and those with cancer (Doering et al 1992, Sullivan et al 1992, Mittanack et al 1993). This usually takes the form of an increased frequency of heterozygotes, such as that observed for the HLA-DQ alpha locus in a normal Asian population (Sullivan et al 1992), or sickle cell anaemia (Simpson et al 1991). In contrast the c-erb B2 polymorphism in c-erb B2 amplified cancers shows a reduction in heterozygotes and increase in homozygotes. The biological basis for selection

pressure usually involves change of gene product function, and at the sickle cell anaemia locus results in heterozygote vigour, but in many other cases is unknown. Polymorphisms or mutations which can alter the normal function of a gene product are usually contained within coding exons of the gene. However, the c-erb b2 polymorphism studied here is contained within an intron (Hall et al 1990a). This does not necessarily mean that this polymorphism cannot effect the gene product as some intronic sequences can have regulatory functions (Kawashima et al 1992). However, a direct functional relationship between genotype and gene amplification is unlikely as c-erb B2 gene amplifications are present in all genotypes. Other oncogenes prone to gene amplification, such as N-myc, can also show a non-random distribution of genotypes, and like c-erb B2, this polymorphism is also contained within an intron (Waber et al 1991). Genetic linkage to another gene of relevance to gene amplification could be another reason for genotype distribution disturbance.

A major finding of this study is that one or both alleles can be amplified. This suggests that there may be more than one mechanism of gene amplification or that different post-amplification events occur. Current theories of how genes become amplified favour a gene recombination event followed by unequal distribution of the recombined DNA into two daughter cells (Stark 1989), one containing gene amplification the other, gene loss (Stark 1989), repetition of the process during subsequent cell cycles could result in multiple gene copies involving both alleles. Mechanisms which amplify single alleles may include amplification after allele loss or allele duplication, a mechanism demonstrated *in vitro* for the c-Ha-ras gene in nitrosomethylurea induced mammary adenocarcinoma (Aldaz et al 1992). Gene overexpression may play a role in these mechanisms (Kraus et al 1987, Shen et al 1988), but experimental evidence *in vivo* has not been obtained.

Examination of the relative intensities of heterozygous c-erb B2 amplified cancers provided further indication of specific allele involvement. One allele was more frequently amplified than both (see Table 25), with a bias towards allele A2. These results indicate that there is a possible association between c-erb B2 allele A2 and c-erb B2 gene amplification. However the exact mechanism(s) of c-erb B2 gene amplification are not clear, and further experimentation is necessary to resolve this.

A potentially confounding factor pertinent to both genotype distribution and allele specific amplification is the presence of loss of heterozygosity at 17q, previously reported in up to 60% of breast cancers (Cropp et al 1990, Devilee et al 1989). This could partly explain a reduced frequency of A1/A2 heterozygotes and an increased frequency of A1 and A2 homozygotes. If this does occur then this would suggest that allele loss occurs more frequently in c-erb B2 amplified cancers and could be related to the amplification process. Such allele losses may be indicated by a deviation of genotype distribution, as allele frequencies would not deviate from normal if the losses were random (equivalent numbers of A1 and A2 alleles would be lost).

An indication of chromosome (therefore allele) loss may be obtained from the analysis of genotype distribution with DNA ploidy change. Aneuploid cancers deviate from the Hardy-Weinberg equilibrium and show a reduction in A1/A2 heterozygotes, and an increase in A1 and A2 homozygotes over those expected. This indicates that some random loss of chromosome 17 DNA may occur, but due to the relatively crude nature of DNA ploidy analysis more specific analysis is not possible. Due to the small size of the study set it was not possible to determine whether the genotype deviation found for c-erb B2 amplified cancers was independent of aneuploidy. However, analysis of DNA ploidy and c-erb B2 gene

amplification (Chapter 4) indicates that aneuploidy is not positively associated with amplification, and diploid cancers are more frequently amplified.

These results clearly indicate that allele specific amplification is worthy of further analysis. Deviation to the expected c-erb B2 genotype distribution in c-erb B2 amplified cancers may indicate a biological basis for amplification mechanisms, either as increased susceptibility or by linkage to another relevant DNA sequence. I have shown that c-erb B2 gene amplification may not be a single or straightforward mechanism, and that different mechanisms or post amplification events occur. Allele loss may be implicated in the amplification process. This is an important area of research as very little is known of the mechanisms of gene amplification which is a common event in breast cancer.

CHAPTER 6

THRA1 - a co-conspiritor?

6.1 INTRODUCTION

Chapter 3 established that c-erb B2 was frequently disregulated in breast cancer, with a high percentage of cancers containing c-erb B2 gene amplifications. DNA amplifications encompassing c- erb B2 on chromosome 17 appear to be variable in size, and may include other important genes nearby (Keith et al 1993). The extent and type of any chromosome 17 DNA amplification within individual breast cancers with c-erb B2 gene amplification is often not known, but may include genes such as the thyroid hormone receptor gene (THRA1), retinoic acid receptor, and topoisomerase II, which have been mapped to 17q13-q21 (Mattei et al 1988, Tsai-Pflugfelder et al 1988, Weinberger et al, 1986). Indeed all four of these genes have been shown to be co-amplified with c-erb B2 in a small number of breast cancers (Keith et al 1993), indicating that amplicon involving c-erb B2 can include several genes. Amplification of THRA1 gene has been reported in 23-64% of breast cancers with c-erb B2 amplification, but not in breast cancers without c-erb B2 amplification (Futreal et al 1992, Tavassoli et al 1989, Tsuda et al 1989). Furthermore, this region of chromosome 17 is of extreme interest in breast cancer as one of the genes responsible for familial breast cancer BRCA1 localised at 17q12-q21 (Hall et al 1990b, Miki et al 1994, Narod et al 1991).

THRA1 is of particular interest as its oncogene, v-erb A, along with v-erb B, is present on the avian erythroblastosis virus (Graf and Beug 1983). V-erb A alone does not appear to transform cells, but when co-transfected with v-erb B, enhances the transforming ability of v-erb B (Frykberg et al 1983, Graf and Beug 1983, Kahn et al 1986). V-erb A does contain several mutations likely to effect the functioning of the receptor (Sap et al 1989), including a missence mutation in the zinc finger DNA binding domain (Bonde et al 1991). These mutations may alter the binding

specificity and abrogate the receptors capacity to bind thyroxine (Bonde et al 1991, Damm et al 1989). No mutations have been found in its cellular counterpart (Futreal et al 1994).

The normal product of THRA1 is the thyroid hormone receptor (TR), a member of the steroid receptor family (Sap et al 1986), which when bound with ligand can act as a transcription factor (reviewed in King 1992). The transcriptional specificity of THRA1 is dictated by the binding affinity of the receptor protein to the steroid response elements (SRE) within the target gene promoter (King 1992). The SRE usually consists of two hexameric DNA base sequences, which may be separated by three variable nucleotide bases within the promoter region of the target gene (King 1992). These sites are recognised by the zinc finger regions of the receptor, which in THRA1 are coded by exons 4 and 5 of the gene (Laudet et al 1991). The amino acid sequence of these zinc fingers is critical for binding the specific target sequences, as only a single amino acid change, Gly-Ala, could convert the receptor into a molecule which would recognise oestrogen rather than thyroxine response elements (Glass et al 1990).

The similarity of oestrogen receptor (ER) and TR in target DNA binding, for both the amino acid sequence of their zinc fingers and the steroid response elements in target genes, suggests that there may be some possible interrelationship between their functions. Mutations in either of these elements could result in an altered THRA1 receptor specificity, which could have the potential to mimic ER specificity. This may be of relevance to cancers with amplified c-erb B2, as one function of oestrogen receptor may be to down regulate the expression of c-erb B2 (Chapter 4, Antoniotti et al 1994, Russell and Hung 1990). Co-amplification of THRA1 may indicate the potential for increased transcription of the receptor, which could potentially effect

the relationship between c-erb B2 amplification and c-erb B2 expression. The DNA binding sequence (zinc fingers) of THRA1 is therefore worthy of investigation. A hypothesis pertaining to possible relationships between THRA1, ER and c-erb B2 is outlined in Figure 43.

The aims of this chapter were to:

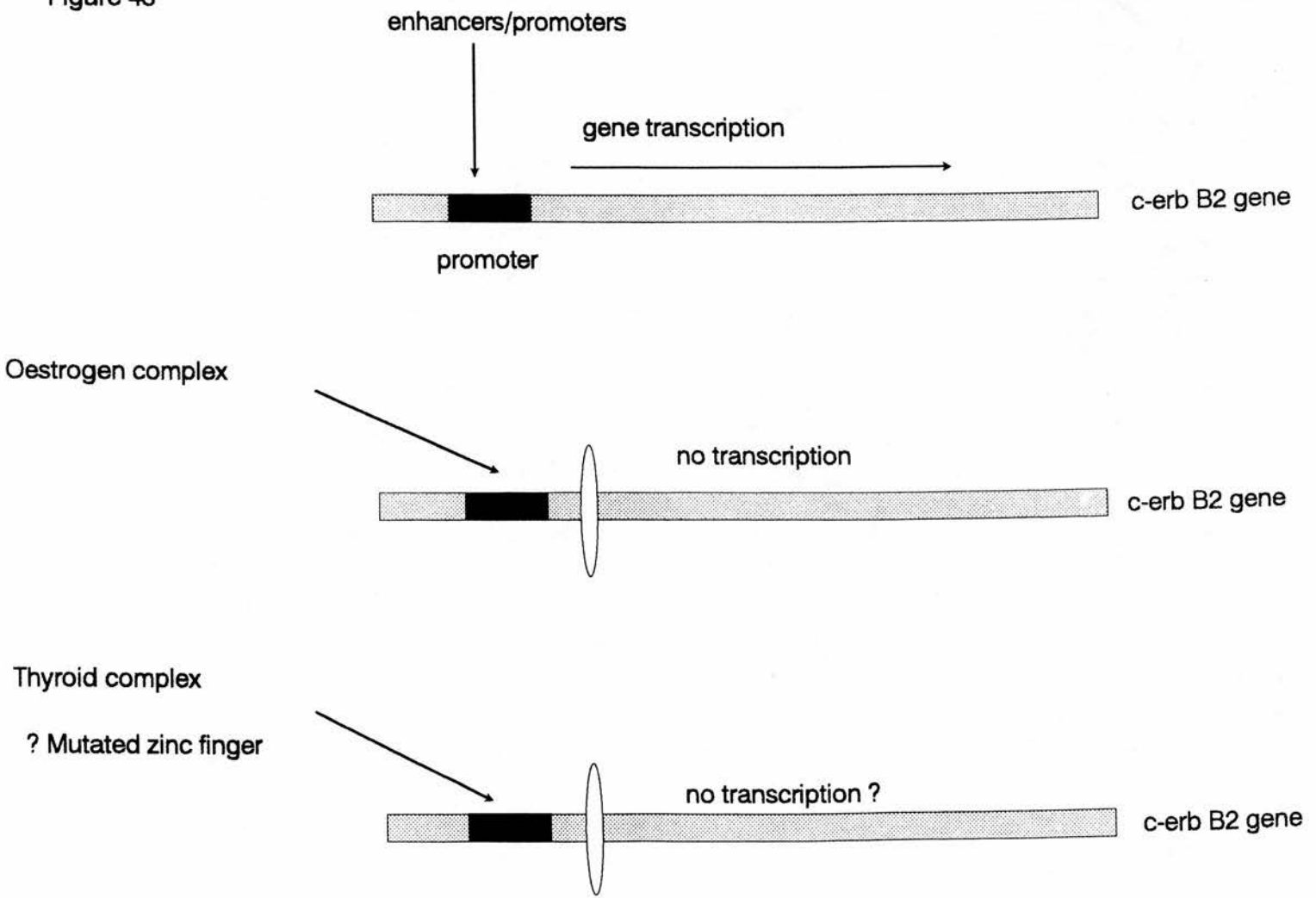
1) Establish the extent of co-amplification of THRA1 and c-erb B2 using differential PCR.

2) Investigate the DNA sequences coding for the zinc fingers of THRA1 for mutations using single stranded conformational polymorphisms (SSCP) and direct nucleotide sequencing.

Figure 43

Ideogram illustrating potential transcriptional control mechanisms of c-erb B2. C-erb B2 gene transcription can be initiated by promoter molecules, possibly in conjunction with enhancer elements. Transcription of c-erb B2 may be blocked, either directly or indirectly, by oestrogen complexed with oestrogen receptor. The thyroid hormone receptor (THRA1) with or without thyroxine, due to close homology with oestrogen binding specificity (zinc fingers), may also be able to block c-erb B2 transcription. This may require the presence of mutations within the zinc finger regions of THRA1.

Figure 43


enhancers/promoters
gene transcription
c-erb B2 gene
promoter
Oestrogen complex
no transcription
c-erb B2 gene
Thyroid complex
? Mutated zinc finger
no transcription ?
c-erb B2 gene

6.2 MATERIALS AND METHODS

6.2.1 Breast Cancer Study Set.

The status of THRA1 was examined in 90 breast cancers, a random subset of the study set previously described in Chapter 3 (section 3.2.1), and in 24 control DNAs derived from normal colon tissue. Template DNA was prepared from frozen tissue and or fixed paraffin embedded tissue according to methods outlined in Chapter 2 (section 2.2.2).

6.2.2 Primers and the Differential Polymerase Chain Reaction

Primers were designed using "Primer" software v0.5 (Whitehead Institute, USA) from the THRA1 DNA sequence published by Laudet et al 1991. DNA sequence was available only for exons 1 to 10, and included only limited flanking intronic sequence. Primers were designed to amplify exons 4 and 5, which represent two zinc finger coding sequences (Laudet et al 1991), and are listed in Table 26.

Primer sets for each exon were first tested individually for specific product in a standard PCR reaction (Chapter 2), but using variable buffer concentrations and annealing temperatures. Primers for exon 4 and exon 5 were then tested for efficiency in the standard differential PCR (Chapter 2, section 2.2.3.2), and ratio values calculated according to methods outlined in Chapter 2 (section 2.2.4). Correction factors of x1.06 and x1.8 were applied to average cpm of exon 4 and IFNG150 in dPCR for exons 4 and 5 respectively.

Table 26.

Primer sequences designed using "Primer" software (Whitehead Institute, USA) for PCR amplification of DNA sequences of exons 4 and 5 of THRA1. The primer sequence, gene location according to base pair numbers of Laudet et al (1991), and PCR product size (in base pairs) are given. PCR product sizes for primer combinations are as follows; A1/A2 =149bp, B1/B2 =126bp, A1/C2 =139bp, B1/C2 =134bp, D1/D2 =235bp

Table 26

Exon	primer	primer sequence	location
4	A1 forward	CTT TGG GCA AAT TGC TTC AT	301-320
	A2 reverse	CTT TGG GCA AAT TGC TTC AT	449-429
4	B1 forward	CCC AGG GTA TAT CCC TAG TT	306-325
	B2 reverse	ATC TCA CCA AGC TTC CAT AC	431-411
4	C2 reverse	ATT GCT TCA TCA TCT CAC CAA GC	439-420
5	D1 forward	TTG GTT CAG GAA GGG GAA G	197-215
	D2 reverse	TCT ATT CCC TCC ACC TGG G	431-413

2.2.2 Single Stranded Conformational Polymorphism (SSCP) Analysis.

DNA from breast cancers and control tissues were examined for mutations or variant sequences in exons 4 and 5 by SSCP. For each sample, 200ng of DNA was PCR amplified in x1 reaction buffer (BCL or Bioline) containing 5mmol $MgCl_2$ (exon 4) or 1.5mmol $MgCl_2$ (exon 5), 200mmol dNTPs, 250pmol primers B1 and B2 (exon 4), or D1 and D2 (exon 5), and 1 unit Taq polymerase (BCL or Bioline). 5% of DMSO was added to PCR reactions for exon 4. Cycling parameters were one cycle of 94°C for 4min, 55°C for 1min, 72°C for 1min, 28 cycles of 94°C for 1min, 55°C for 1min, 72°C for 1min, and one cycle of 94°C for 1min, 55°C for 1 min, 72°C for 5 mins. PCR products were extracted once in an equal volume of chloroform: isoamyl alcohol (24:1) and denatured in 0.3MNaOH, 1mM EDTA at 50°C for 5mins. 7.5 µl of stop loading buffer (Appendix 1) was added to 15µl of denatured PCR product prior to loading on 6% nondenaturing polyacrylamide gel (MDE, AT Biochem Inc.) (see Appendix 1). Each sample was electrophoresed at 20W per gel for either 2 hrs at 20°C or 4.5hrs at 4°C. For gels electrophoresed at 4°C glycerol was omitted from the acrylamide gel solution. To visualise single stranded product bands the gels were stained using Biorad silver staining reagents and protocols, see Appendix 2. After staining the gels were fixed twice in methanol:acetic acid for 5mins and dried under vacuum, then laminated. Band patterns were assessed visually.

6.2.3 Sequencing of DNA with Variant SSCP Bands.

Variant sequences found by SSCP analysis were sequenced using two different techniques: Cycle sequencing and Sequenase sequencing. Template preparation,

resulting in single stranded template for sequencing, consisted of either Dynabeads preparation (Dynal, Norway) or cloning into plasmid vector, pGEM 7z f+.

6.2.3.1 Dynabead Preparation of DNA Template for Sequencing. Dynabeads are superparamagnetic polystyrene beads with streptavadin covalently attached to the bead surface. The beads will bind to biotinylated PCR product, only one strand of which is biotinylated. When the DNA is denatured, the single stranded biotinylated sequence remains attached to the bead and can be magnetically separated from the non-biotinylated strand, thereby providing pure single stranded template for sequencing.

DNA for sequencing was PCR amplified as previously described using a biotinylated primer D1 (Cruachem Ltd, Glasgow). PCR products were extracted with an equal volume of chloroform:isoamyl alcohol (24:1) to remove residual paraffin oil. PCR products were purified on either a Wizard DNA clean up column (Promega, UK Ltd) or G50 sephadex columns (BCL, UK Ltd) according to the manufacturer's instructions.

Twenty microlitres of Dynabeads were washed twice in 5mM Tris pH7.6, 1mM EDTA and 1M NaCl (wash solution), and added to 40µl of purified DNA product and left at room temperature for 15 mins. The Dynabead bound PCR products were separated from the supernatant fluid using a magnet and washed twice in 100µl of wash solution. DNA attached to the beads was denatured by suspension in 8µl of 0.1M NaOH and incubated at room temperature for 10 minutes. After magnetic separation, the supernatant containing the antisense strand was removed. Denaturation was repeated once. The biotinylated strand attached to the Dynabeads

was resuspended in 10µl of double distilled water, this preparation was used directly in sequencing reactions.

The non-biotinylated strand collected in the denature supernatant described above was precipitated in 0.1 volume 5M sodium acetate and 4 volumes of ethanol at -20°C overnight. The DNA precipitate was spun for 10mins at 12,000g, washed once in 70% ethanol and dried under vacuum. The final DNA pellet was resuspended in 10µ l of double distilled water and used directly in sequencing experiments.

6.2.3.2 Preparation of Sequencing Template by Cloning into pGEM 7Z f +.

High concentrations of pure DNA template can be prepared by ligating a blunt ended DNA fragment into a linearised plasmid vector, transforming *E. coli* (*Escherichia coli*) cells, and preparing plasmid DNA from the resulting cultures.

Exon 5 DNA was amplified in a standard PCR reaction. The products were extracted once in an equal volume of chloroform:isoamylalcohol (24:1) and PCR precipitated in 0.1 volume 5M sodium acetate and 2 volumes ethanol at -20°C overnight. The DNA precipitate was spun for 10mins at 12,000g, washed once in 70% ethanol and dried under vacuum. Blunt ends were formed by digestion at 30°C for 15 minutes with 3 units Klenow enzyme (BRL, UK Ltd), x1 blue palette buffer (Sigma, UK Ltd), and 2nm dNTPs in a total volume of 20µl. The reaction was stopped by addition of 1µl 0.5M EDTA, pH8. The blunt ended template DNA was extracted once in 20µl phenol, and once in 20µl 24:1 chloroform:isoamyl alcohol before purification on a G50 sephadex column, according to the manufacturer's instructions (Appendix 2). Two µg of plasmid pGEM 7Z f+ was linearised by

digestion with 9 units of *SmaI* in a total volume of 20µl at 25°C overnight, followed by purification on a G50 sephadex column.

Ligation. The concentrations of prepared insert and vector were estimated by comparison with standards on agarose containing 0.5ng/ml of ethidium bromide. Vector concentration was estimated as 15ng/µl and both cr127 and cr164 contained approximately 10ng/µl. Four ligation reactions were performed. cr127 and cr164 were both ligated at 10:1 and 3:1 insert to vector ratios, and two control reactions were pGEM with no insert, and pGEM with no insert and no ligase. Ligation reactions contained approximately 200ng of total DNA (insert and vector), x1 T4 ligase buffer, and 2.5 units T4 ligase in a total volume of 20µl. All ligation reactions were incubated at 16°C overnight.

Transformation. Library efficient DH5 alpha cells (*E. coli*) (20µl) were mixed with 1µl of a 1:5 dilution of ligated samples or controls, and incubated on ice for 30 minutes. Bacterial cells were heat shocked for 40 seconds at 42°C then placed on ice. 80µl of SOC (see Appendix 1) was added to the cells and incubated at 37°C for 1 hour shaking at 225 rpm. Transformed cells were cultured on L-amp (see Appendix 1) plates coated with 100µl of 100mM IPTG (isopropyl-B-thiogalactopyranoside, Promega UK Ltd) and 20µl 50mg/ml X-gal (Promega UK Ltd), overnight at 37°C. White coloured colonies of transformed cells containing exon 5 insert were picked and grown in L-broth containing 50µg/ml of ampicillin and incubated overnight at 37°C shaking at 225 rpm.

Plasmid DNA containing exon 5 insert was prepared from 3mls of bacterial culture using the Wizard miniprep protocol (Appendix 2). The purified plasmid DNA was finally resuspended in 50µl ddw, this was used directly in sequencing reactions. The

presence of insert DNA was established by electrophoresis after digestion of a 4µl aliquot of plasmid DNA with 3 units of *Pvu II* at 37°C for 2 hours.

6.2.4 DNA Sequencing

Sequencing reactions were performed on template DNA prepared using Dynabeads or by cloning, described above, using two different sequencing protocols.

6.2.4.1 Sequencing using Sequenase. DNA template was sequenced using Sequenase™ Version 2.0 DNA sequencing kit (United States Biochemical, Ohio). 0.5pmol primer D1 or D2 was annealed to the appropriate strand of template DNA in x1 sequenase buffer at 65°C for 2 minutes, cooling to < 35°C over 30 minutes. Alternatively Dynabead prepared DNA template was heated to 94°C for 5 minutes and immediately cooled on ice. The sequence was then labelled on addition of 1µl of DDT (dithiothreitol), 2µl labelling mix containing dNTPs, 5µCi S35 dATP and 1 unit of sequenase enzyme and incubated at room temperature for 3 minutes. The reaction was terminated by adding 3.5µl of the labelled reaction to 2.5µl of each of 4 termination mixes, each containing a different dideoxy nucleotide base (ddATP, ddGTP, ddCTP, or ddTTP). These reactions were incubated at 40°C for 3 minutes and stopped on addition of 4µl of stop solution (Appendix 1).

6.2.4.2 Cycle Sequencing. Cycle sequencing, using Cyclist DNA sequencing kit (Stratagene, USA), was performed on single stranded template DNA prepared by the Dynabead method. For each reaction a master mix of 7µl DNA template, 1pmol primer D2, 4µl sequencing buffer (BRL), 10µCi S35 and 2 units of Taq polymerase (BRL) and 10µl H_2O was prepared. Reactions containing 3µl of each dideoxynucleotide (ddATP, ddGTP, ddCTP, or ddTTP) and 7µl of master mix were

denatured at 95°C for 5 minutes followed by 30 cycles of 95°C for 30 sec, 55°C for 30sec, and 72°C for 60sec in a Techne PHC4 thermal cycler. The reactions were stopped by the addition of 5µl of stop mix.

6.2.5 Polyacrylamide Gel Electrophoresis

Sets of samples prepared by either of the 2 methods described above were heat denatured at 94°C for 3 minutes, and 3µl was loaded per track on a 6% denaturing polyacrylamide gel (see Appendix 2) and electrophoresed for 1 or 2 hours at 70W. Gels were fixed in 10% methanol 10% acetic acid for 10 minutes, then heat dried under vacuum for 2 hours. Dried gels were exposed to X-Omat x-ray film overnight and developed automatically in a X2 Hyperprocessor (Amersham UK). The DNA sequence was assessed visually.

6.3 RESULTS

6.3.1 PCR of THRA1, Exons 4 and 5.

Exon 4. Exon 4 primer sets A1/A2, B1/B2, A1/B2, B1/B2, B1/A1, A1/C2 and B1/C2 (see Table 26) were tested in dPCR reactions containing 200ng control placental DNA (Sigma, UK Ltd). Reaction buffers varied according to buffer concentration (0.5mM, 1mM, 1.5mM, 2.5mM or 5mM $MgCl_2$) and annealing temperatures (50°C, 53°C, 56°C and 60°C).

PCR with primers A1/A2 failed to produce detectable product at any annealing temperature and $MgCl_2$ concentration combination. Therefore a second set of primers, B1/B2, was designed and the experiment repeated. Low concentration of product was produced from control placental DNA (Sigma) with these primers at an annealing temperature of 54°C, and $MgCl_2$ concentrations of 2.5 and 5mM. Amplification efficiency was not improved by the use of primers combinations A1/B2, B1/A2, A1/C2 or B1/C2. Examination of each primer concentration showed that B2 appeared x10 less concentrated than B1, although spectrophotometric readings supplied had indicated similar concentrations. After the concentration of B2 was increased ten fold, PCR produced an acceptable concentration of product. All further experiments were conducted with ten fold primer B2.

Exon 5. Primers for exon 5 (D1/D2) were tested in a standard PCR containing 200ng of control placental DNA (Sigma), and produced high concentration of product at an annealing temperature of 55°C and 1mM $MgCl_2$ concentration in the reaction buffer. PCR of template derived from fixed tissue was not as efficient as DNA derived from frozen tissue.

6.3.2 Quantitation of THRA1 Gene Copy Number by dPCR.

Exon 4. The efficiencies of primers B1/B2 (exon 4) and IFNG150 were tested in dPCR using control placental DNA (Sigma) and breast cancer cr111 as DNA template. A dPCR was performed using 50pmol, 100pmol, 200pmol, 250pmol, and 500pmols of primers B1 and B2(exon 4), with 250pmol of IFNG150 in the standard PCR reaction, at an annealing temperature of 55°C and PCR product measured as described in Chapter 2. Increasing concentrations of primers B1 and B2 did not effect dPCR ratio values obtained, indicating abnormal reaction kinetics. Because of this finding and the problems encountered with primer concentrations (above) I decided to concentrate on exon 5 for dPCR.

Exon 5. Primer efficiencies of D1/D2 (exon 5) were tested in a dPCR using 200ng of control DNA (Sigma) and cr111 as DNA template. A dPCR was performed using 50pmol, 100pmol, 150pmol, 250pmol and 500pmol of D1/D2 primers with 250pmol IFNG150 primers in a standard reaction at an annealing temperature of 55°C. Ratio values obtained increased with increasing D1/D2 primer concentrations, (Figure 44), therefore an equimolar concentration of primers for D1/D2 and IFNG150 was chosen for further experiments.

To further test primer efficiency dPCR was performed on control DNAs (Sigma and p258) and cancer cr111 in a standard reaction containing primers for TH5 and IFNG150, and PCR product was sampled at 23, 26, 30 and 35 cycles and ratio values calculated. The results indicate that the reaction efficiencies of the primer sets were similar, (Figure 45), although control DNAs (Sigma and p258) showed a trend towards lower ratio values with increasing cycle number. Attempts to "spike" PCR

Figure 44

Titration of THRA1 exon 5 primer (TH5) concentration in differential PCR with 250pmol of IFNG150 (reference gene) primers. DNA template were control DNAs Sigma and p258 and one cancer DNA cr111. Results were expressed are dPCR ratio values and show that as TH5 primer concentrations increase, dPCR ratio values also increase.

Figure 45

Differential PCR cycle course using equimolar concentrations of TH5 and IFNG150 primers in a standard dPCR reaction. PCR product was sampled at cycle numbers 23, 26, 30, and 35. Results are expressed as ratio values.

Figure 44 Titration of TH5 primers in dPCR

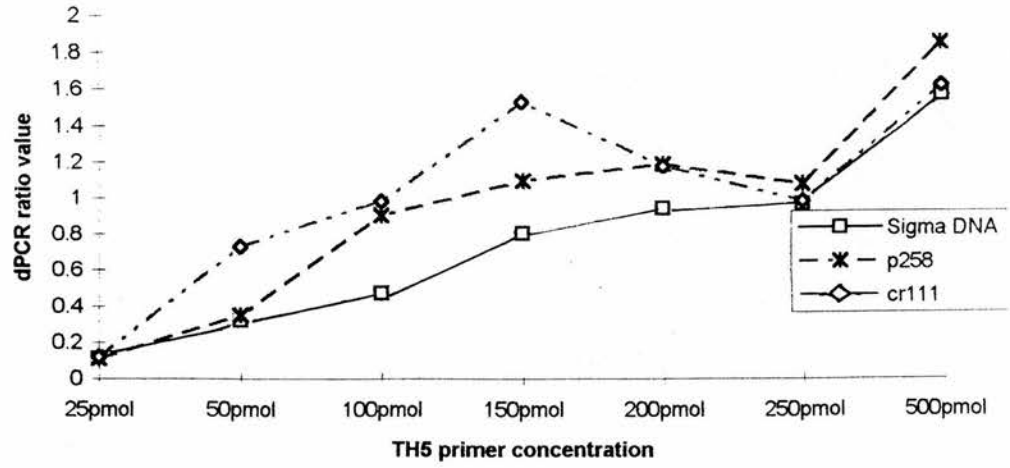
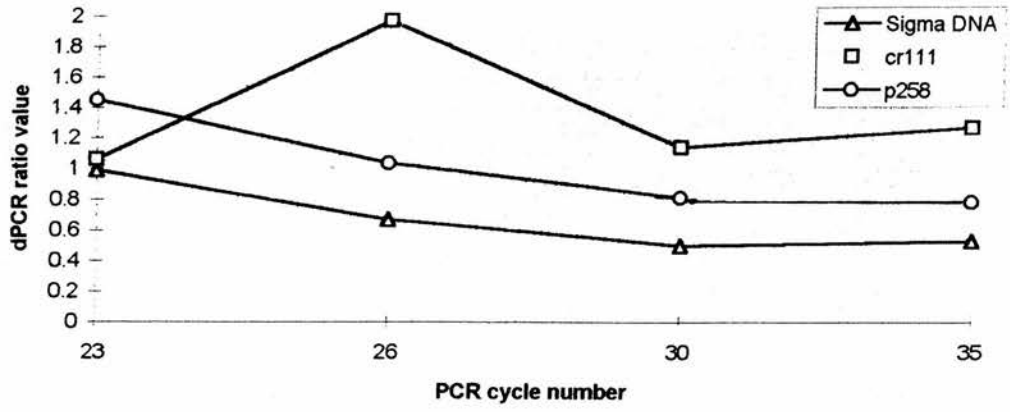


Figure 45 Differential PCR ratio values throughout a thermal cycle courseCycle course TH5



of control DNA with THRA1 PCR product failed to produce "amplified" ratio values.

THRA1 dPCR in breast cancers. Thirty nine breast cancers, 28 c-erb B2 amplified and 11 non-amplified, were tested for THRA1 gene copy number in a standard dPCR reaction containing 200ng template DNA. The range of ratio values found for amplified cancers (0.56-1.82) was similar to those found for non amplified cancers (0.69-1.38). Repeat tests found poor consistency in the ratio values found for individual samples.

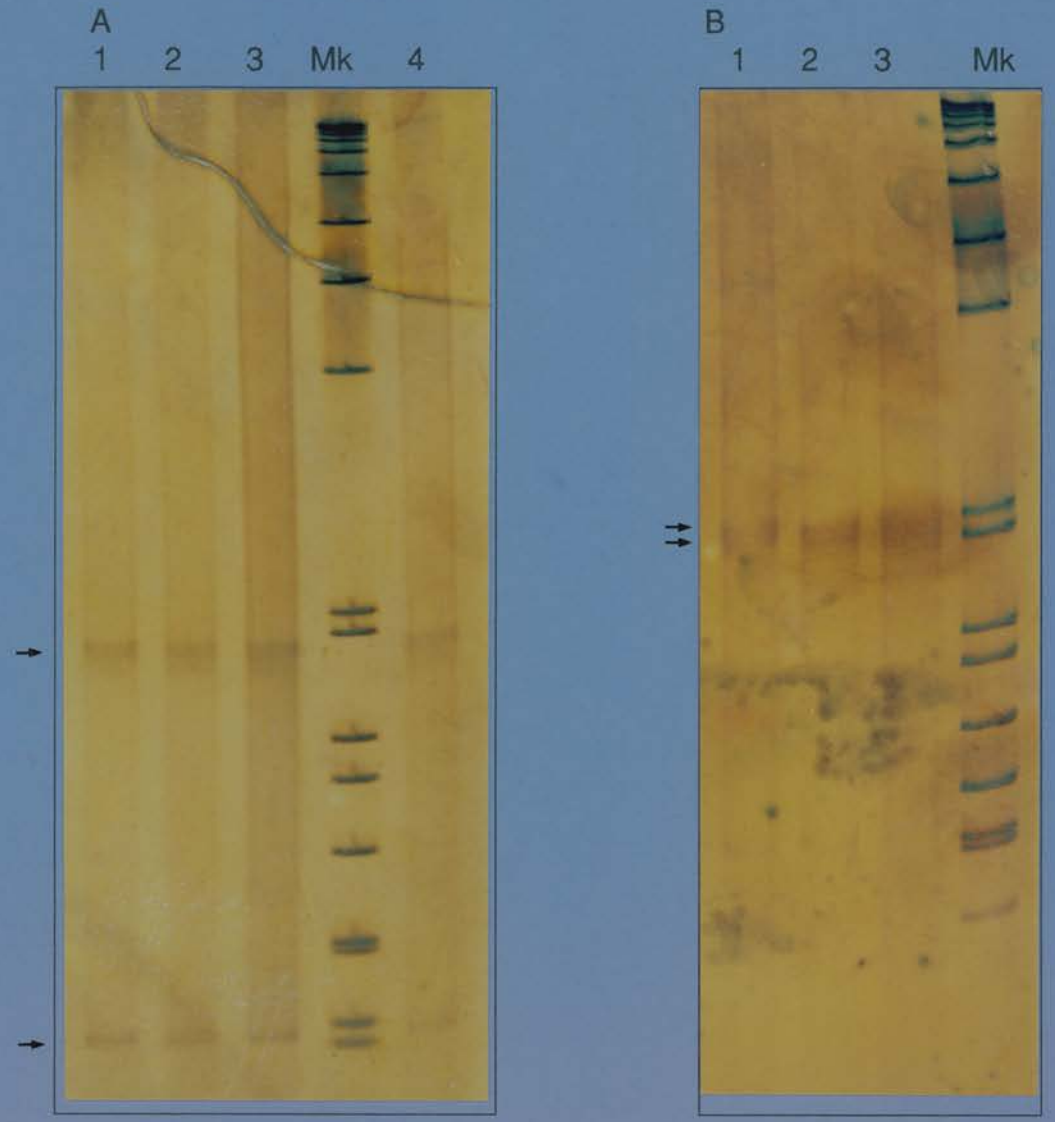
6.3.3 SSCP

Exon 4. 64 breast cancers (25 c-erb B2 amplified, 31 nonamplified and 8 not known), and 11 control DNAs were assessed for mutations in exon 4 by SSCP analysis. Gel electrophoresis at 20°C produced 3-4 bands, and 2 bands at 4°C, which were normally present in all samples, and are illustrated in Figure 46. Analysis was complicated in some samples by the presence of non-specific PCR amplified fragments, which made interpretation of these gels difficult. However, when present, non specific bands were numerous and were unlikely to be mistaken for a mutant band. Nevertheless , it is possible that non-specific bands may have concealed a real mutant band. Attempts to improve the quantity of PCR product in order to increase the signal on SSCP analysis by changing reaction pH and buffering conditions, resulted in more non-specific fragments. Of the cases tested 49 were free of multiple non specific bands, and none of these had any additional bands suggestive of THRA1 exon 4 mutation.

Figure 46

SSCP analysis of exon 4 of THRA1 of PCR amplified sequence from breast cancer DNA. Photographs show the resulting SSCP bands after electrophoresis of a 149bp PCR amplicon on 6% MDE polyacrylamide gel. Electrophoresis was performed at 20°C (A) and 4°C (B). All cancers show similar patterns of bands, (A) tracks and (B) tracks 1-3, indicated by arrows. Marker DNA in each gel was a 1kb ladder (BRL, UK.).

Figure 46



Exon 5. Exon 5 was assessed for potential mutations in 90 breast cancers, (34 c-erb B2 amplified, 36 non amplified, and 18 not known) and 24 control DNAs. Gel electrophoresis at 20°C usually produced 4 major bands, illustrated in Figure 47. A single variant band was detected in 7 cancers and 2 controls, illustrated in Figure 48. This band was of equal intensity to the other major bands and was not present, even faintly, in any of the other cancers or controls. SSCP analysis at 4°C failed to detect any additional variant bands.

Clinical and biological features of 7 cancers producing a variant band are listed in Table 27. Cancer diameter ranged in size from 20mm to 50mm. All were negative for c-erb B2 expression, but 4 were c-erb B2 amplified. Oestrogen receptors ranged from 0 to 344fmol/mg protein, but were generally low with 5 below 50 fmol/mg protein. All cancers with abnormal THRA1 bands were from the non-screened group.

6.3.4 DNA Sequence of SSCP Variants.

Samples which produced a variant band on SSCP analysis, (cr111, cr127, cr164, and cr569 and 1 colon control, n18) were sequenced to identify the location and type of variant.

6.3.4.1 Dynabead preparation and Sequenase and Cycle sequencing. DNA prepared from breast cancer samples cr111, cr127, cr148, cr216 and normal controls n18 and placental DNA (Sigma) using Wizard or G50 purification methods (see Appendix 2), produced only a partial sequence of exon 5 when sequenced using Sequenase or Cycle sequencing techniques.

Figure 47

SSCP analysis of exon 5 of THRA1 of PCR amplified sequence from breast cancer DNA. Photographs show the resulting SSCP bands after electrophoresis of a 253bp PCR amplicon on 6% MDE polyacrylamide gel. Electrophoresis was performed at 20°C (A) and 4°C (B). At 4oC all cancers showed similar patterns of SSCP bands, (B) tracks 1-4. Electrophoresis at 20°C revealed one varient band (indicated by arrow), illustrated in (A) track 1. Tracks (A) 1-4 were breast cancer cases, and tracks 5-7 were controls. Marker DNA in each gel was a 1kb ladder (BRL, UK.).

Figure 47

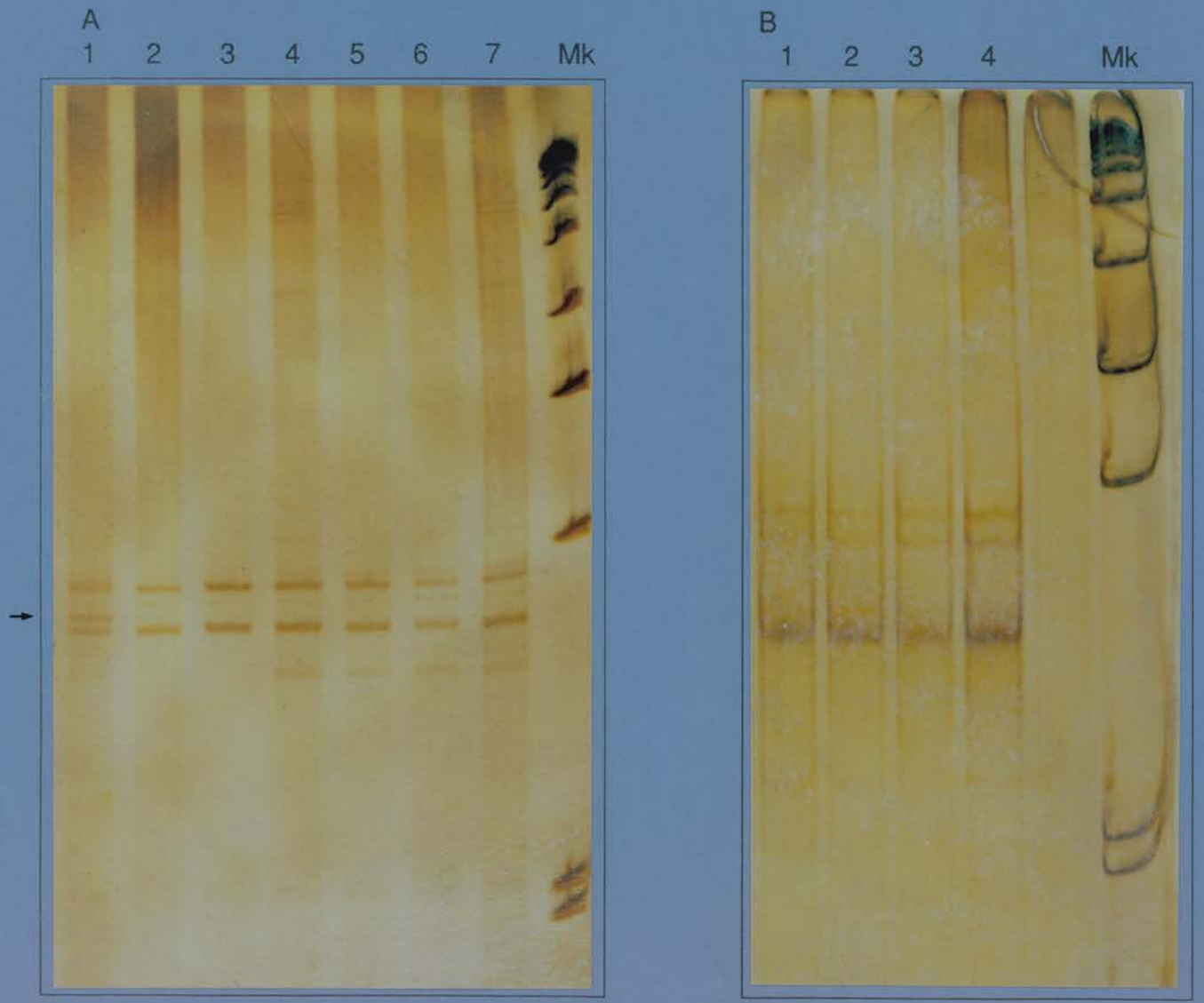


Figure 48

SSCP analysis of exon 5 DNA at 20°C. Tracks 1-3 represent three different breast cancers all containing a single varient band (indicated by arrow). Tracks 4and 5, and 6 and 7 represent two control DNAs with matched colon cancer DNA, all of which exhibit the varient band. Tracks 8 and 9 contain breast cancer DNA, and tracks 10-13 contain control DNA, none of which exhibit the varient band. Marker 1 (Mk1) is BRL marker V, and marker 2 (Mk2) is 1kb ladder (BRL, UK).

Figure 48

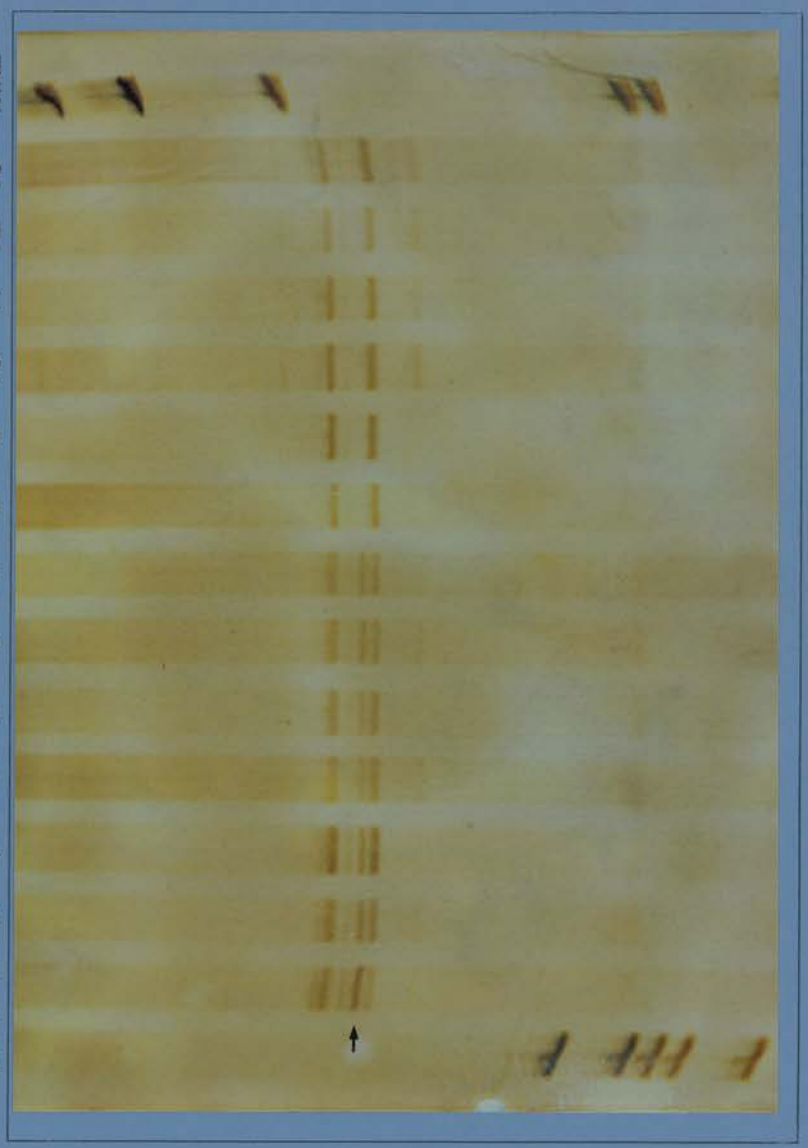


Table 27

SSCP analysis detected a variant DNA sequence in seven breast cancers. Histopathological and biochemical features (described in Chapter 4) of these cancers are given in this table. DCI NST = ductal carcinoma of no special type, MCI = special type invasive cancer (mucinous). Cancer grade (I, II, or III) was determined according to the method of Elston (1987). DNA ploidy was defined as DIP = diploid, AN = aneuploid, and TET = tetraploid. Lymph node status defined positive if cancer cells had metastasised to the nodes.

Histopathological Cancer Type

DCI NST	**6**
MCI	**1**

Histopathological Cancer Grade

Grade I	**2**
Grade II	**2**
Grade III	**3**

DNA Ploidy Status

DIP	**2**
AN	**5**
TET	**0**

Lymph Node Status

node positive	**1**
node negative	**6**

The DNA sequence of a 60 base pair section at the 3' end of the PCR fragment, consisting of part of intron 5 up to the start of exon 5 was identified, but contained no variant DNA sequence. The 148bp sequence of DNA coding for the exon and 27 bases of intron 4 were unreadable due to "stops" where bands were present in all four tracks.

Attempts to solve this problem included alteration of the annealing temperatures, termination temperature, and reaction times of labelling and termination reactions, and addition of DMSO to annealing and termination reactions. None of these alterations produced any improvement on the length of readable sequence. Similar problems were encountered when the reverse strand was sequenced. In an attempt to increase specificity and reduce possible secondary structure cycle sequencing was performed on cr127 and cr164. The higher temperatures involved in the PCR cycles failed to reduce the possible impact of secondary structure and no additional sequence could be read.

6.3.4.2 Plasmid cloning and Sequenase sequencing. PCR amplified DNA of exon 5 from breast cancers cr127 and cr164 was cloned into pGEM 7f+ and transformed DH5 alpha cells (*E. coli*) prior to sequencing. White colonies were present in all ligation reactions (3:1 and 10:1 insert:vector), signifying the presence of transformed DH5 alpha cells. Control plates of pGEM plus ligase only produced blue colonies, pGEM minus ligase produced no colonies. Five white colonies, containing plasmid and insert DNA, from each ligation reaction of cr127 and cr164 were cultured overnight, and plasmid DNA prepared. Restriction digestion of plasmid DNA with *Pvu II* showed the presence of insert DNA but two different patterns of bands, illustrated in Figure 49.

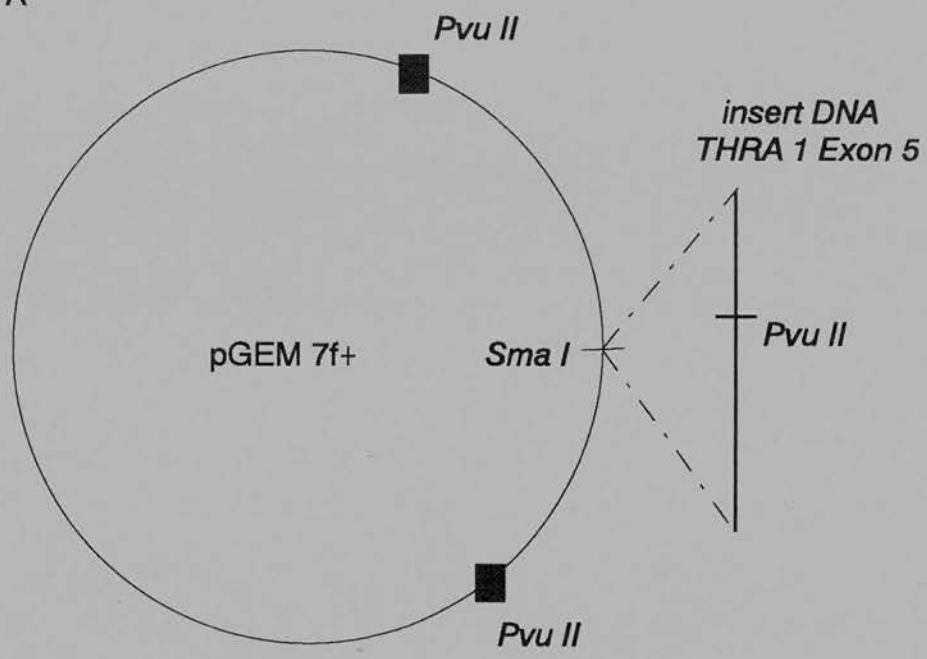
Figure 49

A. Diagram of plasmid vector pGEM 7f+, showing the *Sma I* cloning site, and *Pvu II* restriction sites, which were used to check for correct insert DNA.

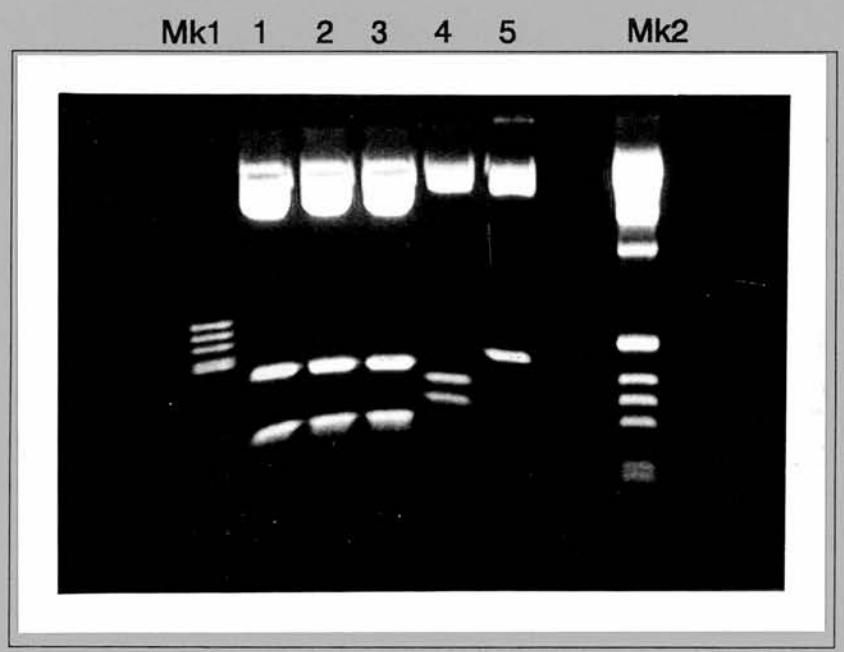
B DNA prepared from four different transformed colonies are shown in tracks 1-4, and represent DNA inserts from PCR reactions from breast cancer cases cr127 (tracks 1-2) and cr164 (tracks 3-4). Tracks 1-3 show the expected restriction fragments from the correct exon 5 insert. Track 4 represents an unknown insert fragment. Track 5 shows *Pvu II* restrictiion of pGEM 7f+ without insert DNA. Marker tracks are Mk1 = BRL marker V, and Mk2 BRL kb ladder.

Figure 49

A


Pvu II
insert DNA
THRA 1 Exon 5
pGEM 7f+
Sma I
Pvu II
Pvu II

B


Mk1
1
2
3
4
5
Mk2

The plasmids containing DNA inserts were sequenced using both forward and reverse primers D1 and D2. A complete sequence was obtained from 6 of the 10 selected colonies, four of which had a sequence indistinguishable from the published sequence, Figure 50. Two clones produced a totally different sequence when the reverse primer D2 was used in sequencing, however no sequence was detected with the forward primer, D1, indicating that these were non-specific PCR products.

A single base transition from C to T was identified in a single clone of both CR127 and CR164, Figure 50. The transition occurs within the coding sequence of the second zinc finger of THRA1, and converts GCC to GCT, Figure 51. Both these triplets code for arginine, therefore this variant is silent. Other clones of cr127 and cr164 contained the normal DNA sequence, indicating that these cancers were heterozygous at this locus.

Figure 50

DNA sequence of exon 5 of THRA1 from two different breast cancers, each exhibiting a varient band on SSCP analysis.

I Sense strand sequence for breast cancers cr127 and cr164. This sequence does not deviate from normal.

II Antisense strand sequence for two clones of cr127. The normal sequence is shown in cr127 A, and the varient sequence is shown in cr127 B. The single base change is indicated by an arrow.

Figure 50

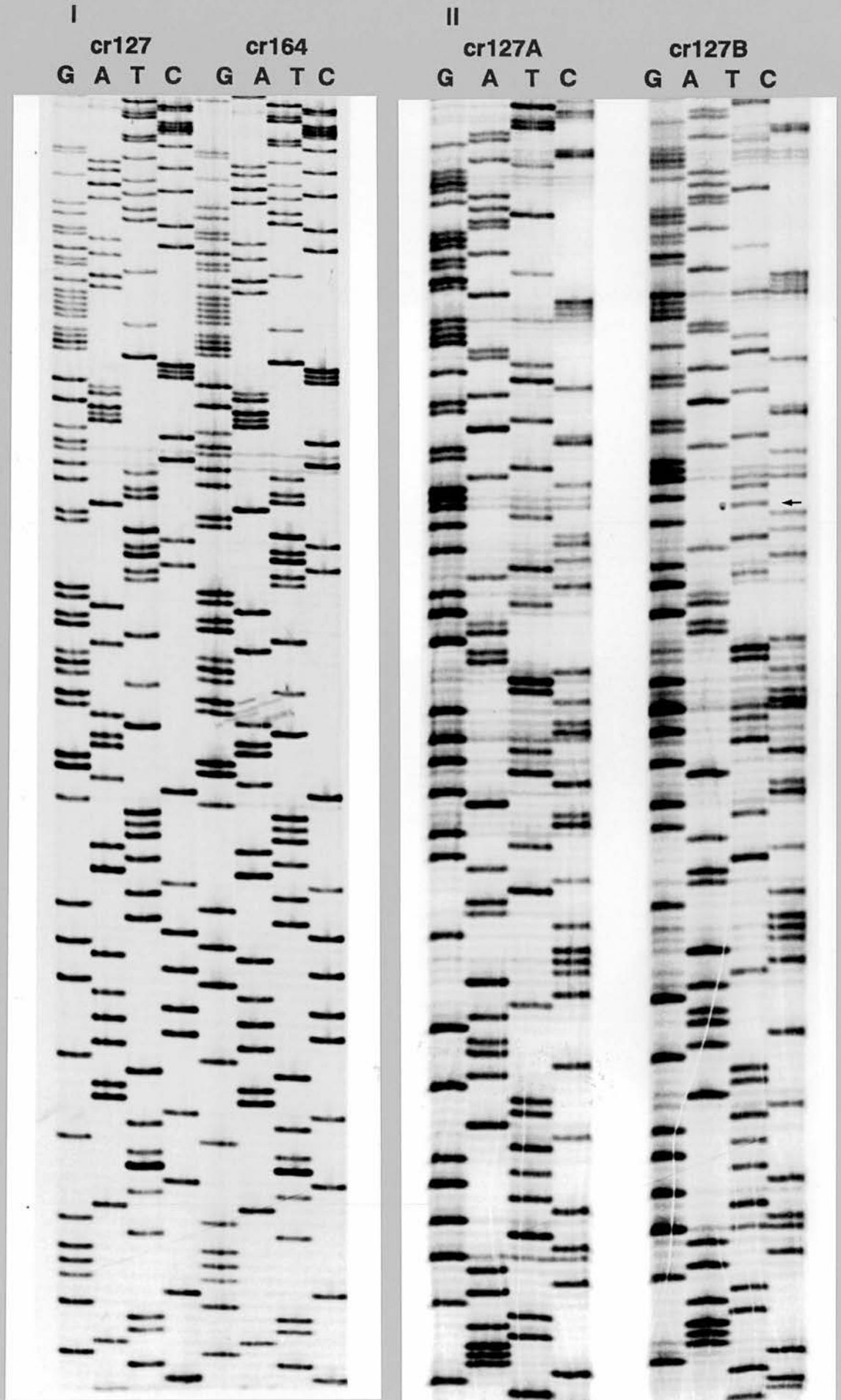
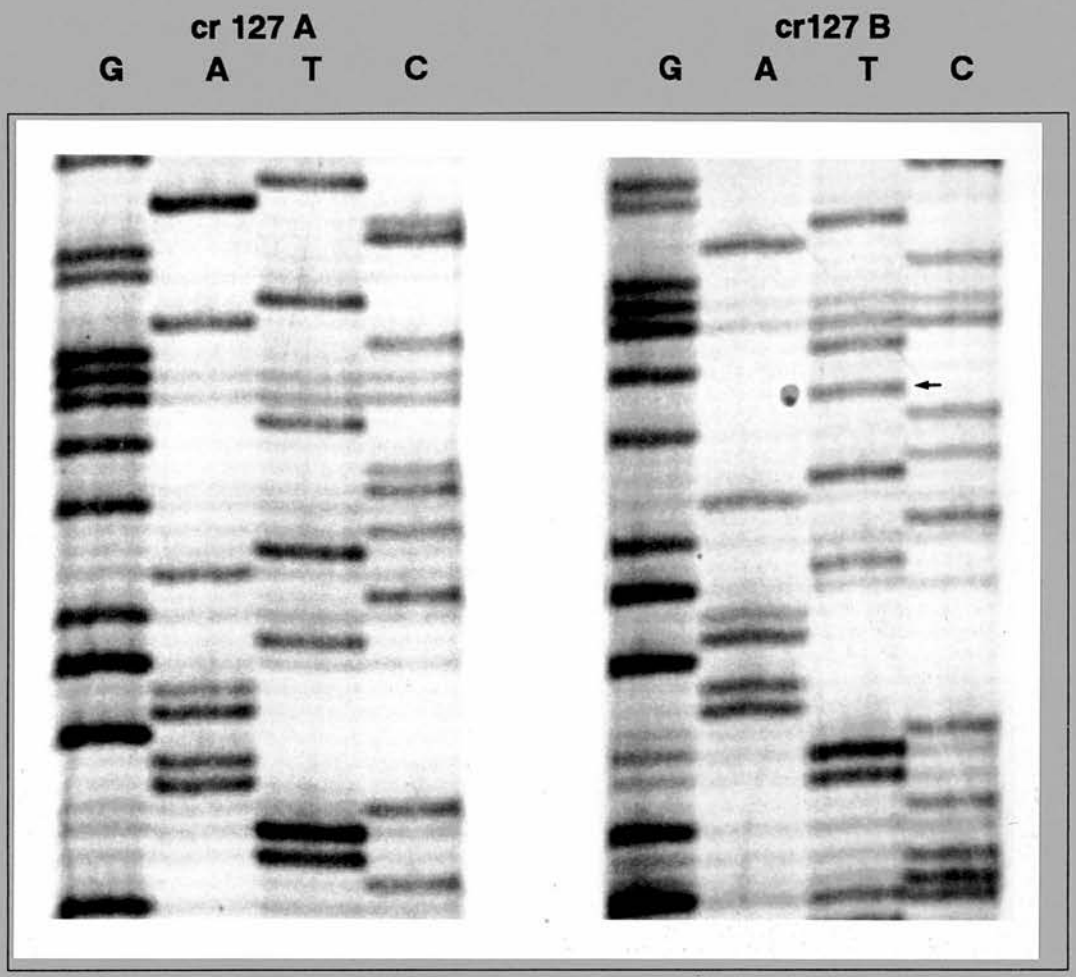


Figure 51

Enlargement of DNA sequence in Figure 49 II, showing the single base transition from C to T, indicated by arrow.

Figure 51



6.4 DISCUSSION

There are currently no convincing data that THRA1 plays a role in human breast carcinogenesis. In experimental systems co-transfection of v-erb B and v-erb A indicates that v-erb A has a synergystic effect on cancer cell growth (Frykberg et al 1983, Kahn et al 1986, Graf and Beug 1983). Mutations play an important role in the transforming capabilities of v-erb A, and mutations can alter the binding specificity and the ability of the gene to promote transcription (Bonde et al 1989, Damm et al 1989, Sap et al 1989). Several mutations, associated with generalised thyroid hormone resistance disorder, have been identified in c-erb A beta (Parrilla et al 1991), however no mutations have so far been identified in c-erb A alpha (THRA1) (Futreal et al 1994). Similarly, co-amplification of THRA1 has not been observed in the absence of amplification of c-erb B2 (Futreal et al 1992, Tavassoli et al 1989, Tsuda et al 1989). Nor have previous attempts to measure transcription in breast cancer tissue proved successful (Tavassoli et al 1989), though this study may have been limited by technical factors such insensitivity, a short half life of the nuclear receptor, or that THRA1 mRNA is naturally present at low copy numbers (5-10 copies per cell) in many adult tissues (Sap et al 1986).

In this chapter I addressed the postulate that co-amplification of THRA1 could be of relevance to the biology of breast cancer and that mutations within the zinc finger coding sequences of THRA1 could change the specificity of the receptor, possibly mimicking the specificity of oestrogen receptor. Amplification of THRA1 was not demonstrated for technical reasons, but no significant mutations in the critical exon 4-5 (Zinc finger) region of the gene were found . The only abnormality detected in this region turned out to be a polymorphism involving a conservative base change.

This polymorphism was not detected in a previous study (Futreal et al 1994), possibly due to the low frequency of this allele and that only 20 breast cancers were investigated. It remains possible that mutations in other parts of the gene , not examined here, could influence the normal function of the receptor protein (Koenig et al 1989).

Hence, although the power of this study was limited, the data are against THRA1 playing a major role in breast carcinogenesis. THRA1 receptor protein would appear to have normal binding specificity and that the single base pair change found in exon 5 represents an infrequent polymorphism. It remains possible that co-amplification of THRA1 and c-erb B2 may be of some relevance, but further investigation is required to resolve this.

CHAPTER 7

Urokinase Plasminogen Activator: An Effector of Aggression in Breast Cancer?

7.1 INTRODUCTION

Analysis of c-erb B2 gene expression and amplification with various cancer characteristics in chapters 3 and 4 has suggested that disregulation can occur at an early stage of progression. Disregulation of c-erb b2 has also been associated some cancer characteristics associated with late stage disease and aggression in breast cancer (Borresen et al 1990, Borg et al 1990), a major indicator of this being the dissemination of cancer cells to lymph nodes and metastasis to other organs (Tubiana and Koscielny 1991). There is no evidence for the direct involvement of c-erb B2 in cancer cell dissemination, although co-expression of c-erb B2 and urokinase plasminogen activator (uPA) has been reported in a small number of breast cancers (Del Vecchio et al 1993). This observation has not been tested in a large, well characterised cancer set.

Overexpression of the serine protease, urokinase plasminogen activator (uPA) in epithelial cells has been linked to aggressive behaviour in breast cancer (Duffy et al 1988, Janicke et al 1990). The biochemical action of the enzyme may promote metastasis by degradation of collagen in the tumour's extracellular stroma (Wilhelm et al 1988). The conversion of plasminogen to plasmin by uPA is thought to be a critical step in this degradation (Liotta et al 1981). Plasminogen activators may have functions other than matrix degradation and could include the promotion of expression of growth factors/receptors (Mizoguchi et al 1990).

Previous studies of breast cancers by immunohistochemistry using a monoclonal antibody directed against human uPA have shown overexpression of uPA in all 115

cancers tested (Janicke et al 1990). Analysis of uPA antigen by ELISA, in the cytosolic fraction of these cancers indicated that they contained, on average, 11 times more uPA than normal breast tissue (Janicke et al 1990). High concentrations of uPA in breast cancer tissue may be an independent prognostic factor in predicting early relapse (Duffy et al 1990, Foekens et al 1992, Grondahl-Hansen et al, 1993, Janicke et al 1990).

The mechanisms of overexpression of uPA in breast cancer are not clear and may involve disregulated promoter or suppressor activity, or fundamental DNA changes such as gene amplification. The regulation of the concentration of uPA in the cytoplasm of cells is complex. It can be influenced by a number of cell signals such as hormones (Mira-y-Lopez et al 1983), phorbol esters (Stopelli et al 1986), growth factors (Rorth et al 1990), and cytokines (Neidbala et al 1992). Another possible mechanism for overexpression is gene amplification. Overexpression associated with gene amplification has been demonstrated in breast cancer for some oncogenes, such as c-erb B2 and c-myc (Riou et al 1984, Riou et al 1987, Slamon et al 1987, Slamon et al 1989), although these genes can be disregulated by more than one mechanism.

This chapter examines the expression of urokinase plasminogen activator and its relationship to clinical features of a breast cancer population, in particular whether possible associations between c-erb B2 disregulation and uPA protein expression could occur. I developed a new differential PCR to determine whether overexpression of uPA could be due, in some cases, to uPA gene amplification. Analysis of this gene also provides the opportunity to assess dPCR in a further gene, using the same source material as the initial development of the method in Chapter 2 and 3.

7.2 MATERIALS AND METHODS.

7.2.1 Breast Cancer Study Set.

Samples of tissue were collected from 134 breast cancers, a random subset of the population previously described in Chapter 3. All were primary cancers, which had not been included in neo-adjuvant treatment studies (Anderson et al 1991). Tissues were fixed and processed according to methods outlined in Chapter 2, section 2.2.1. Control tissue was obtained from breast tissue distant from the lesion site or from non-cancer bearing breasts. For cancers, histological type, grade, and node status were assessed according to criteria described in Chapter 4 (section 4.2),and cancer cellularity and ploidy status were determined according to methods described in Chapter 3 (sections 3.2.1 and 3.2.2).

Oestrogen receptors were measured according to the method described in Chapter 4 (section 4.2), from tissue that had been collected, frozen and then stored at -196°C at the time of operation. The concentration of oestrogen receptors in each cancer sample was expressed as a concentration of protein, with values of >20fmol/mg protein considered positive for oestrogen receptor as this was a concentration regarded as clinically significant for treatment with anti-oestrogens (Hawkins et al 1981).

7.2.2 Immunohistochemistry.

Expression of uPA protein was examined with a murine monoclonal antibody, #394, specific for human urokinase plasminogen activator (American Diagnostica Inc. New

York, NY). Four micron paraffin sections of all cancers were dried at 56°C overnight. The sections were dewaxed in xylene and rehydrated to water. Endogenous peroxidase was blocked by exposure to 1% hydrogen peroxide in methanol for 15 minutes before staining. The sections were washed in Tris-buffered saline (TBS pH7.6), followed by a 10 minute incubation with normal rabbit serum (Scottish Antibody Production Unit, Carluke, UK) diluted 1:5 with TBS. The primary antibody was applied at a 1:100 dilution in normal rabbit serum in TBS and incubated at room temperature for 30 minutes. Normal rabbit serum was used in place of the primary antibody as a negative control. An ABComplex method was carried out according to manufacturers instructions (Dako K355). The sections were lightly counterstained in haematoxylin. To score positive, indicating uPA expression cancer cells had to show weak, moderate or strong brown staining of cytoplasm above normal stromal components of the tissue section, excluding macrophages. A positive control , a cancer previously identified as showing strong staining with #394, and a negative control were incorporated in each set of sections for staining. Some variation in staining intensity was noted in control sections. A fall in titre from 1:100 to 1:10 during overnight storage after reconstitution of the lyophilised preparation was observed in 3 separate batches of #394, and hence fresh dilutions of #394 were prepared for each batch of slides.

Immunohistochemistry results were assessed for associations with other cancer characteristics such as histopathological type, grade, lymph node status and ER status, and presented in a tabular format. Results were tested for significance by X2 analysis of contingency tables. Urokinase expression was also examined for associations with c-erb B2 expression and amplification which were previously assessed in Chapter 3.

7.2.3 Primers and the Differential Polymerase Chain Reaction

Primers for differential PCR for uPA were designed from the human uPA gene sequence published elsewhere (Riccio et al 1985), using "Primer" software (Whitehead Institute, USA). The primer sequence for the sense strand was 5' CAGTTTACCCTCACCCTGGA 3' (1631- 1650bp), and the antisense strand 5' AGCCAACTGTTGTAGGGGTG 3' (1757-1738 bp). PCR using these primers yields a 111bp product from the intron 2-exon 3 boundary of the published sequence. The single copy reference gene used in dPCR was a 150bp sequence of human interferon gamma (Frye et al, 1989).

Fixed paraffin breast tissues were prepared for dPCR according to the method described in Chapter 2 (section 2.8.1). Differential PCR was performed using the standard protocol described in Chapter 2, section 2.2.3.2 using 0.25nmol each of the two primers for uPA (described above) and IFNG150. Differential PCR ratio values were calculated according to methods outlined in Chapter 2, section 2.2.4. For uPA a correction factor of 1.04 was applied to compensate for differences in dCTP content between amplicons IFNG150 (69 C bases) and uPA (66 C bases).

7.3 RESULTS

7.3.1 Detection of urokinase plasminogen activator expression by immunohistochemistry.

Immunohistochemistry for expression of uPA protein was performed on 134 breast cancer specimens. Overexpression of uPA was identified by the presence of a brown reaction product in cancer cells and the absence of this stain in normal tissue. Overexpression of uPA protein was observed in 40 of 113 (35%) invasive cancers and in 4 of 21 (19%) *in situ* cancers, (total cancers 33%). Staining was concentrated in the cytoplasm of the cancer cells, and usually present evenly throughout the cancer cell population in each positive tissue, illustrated in Figure 52. Staining of the cell membrane was present in some positive cancers, however this was always in association with cytoplasmic staining. In some cases weak staining was observed in normal stroma and infiltrating lymphocytes, but this was always in association with positively stained cancer cells. Strong staining was observed in cells identified as macrophages in a small number of cases; if present only in macrophages, samples were considered negative.

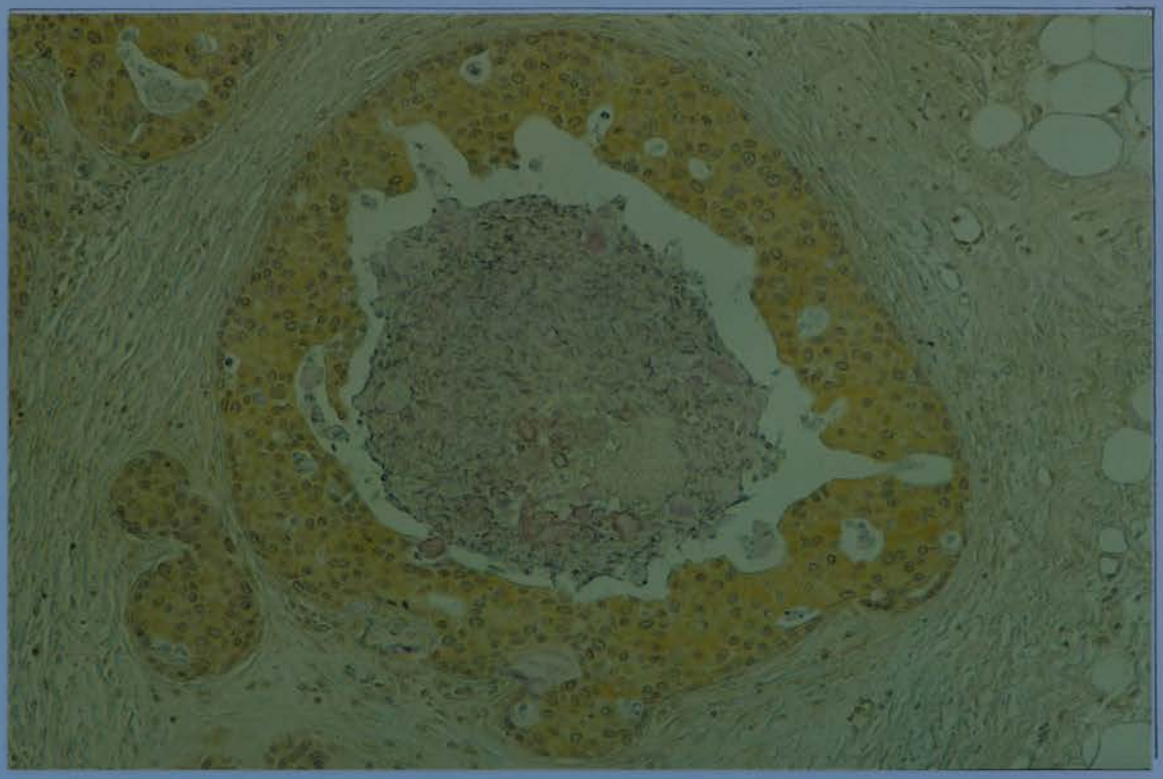
Table 28 summarises the association between uPA protein overexpression and features of the cancers studied. uPA expression was more common in invasive cancers , and within that group was more frequent in oestrogen receptor positive cancers, although neither trend was significant, X2= 0.19, p>0.5. "Positive" staining was present in similar proportions in each of the ploidy groups, diploid (36%), aneuploid (43%) and tetraploid (38%), X2= 0.22, p>0.5. There was no correlation of uPA expression with size of cancer , X2 = 4.38, p=>0.1,<0.5 ,or node status,

Figure 52.

Photomicrograph of four micron section of an *in situ* carcinoma (A) and an invasive ductal carcinoma of no special type (B). Both photographs illustrate strong positive anti uPA staining in the cytoplasm of cancer cells. Normal stromal cells do not stain. Cancer B shows some tissue retraction, an artefact of fixation and processing.

Figure 52

A



B

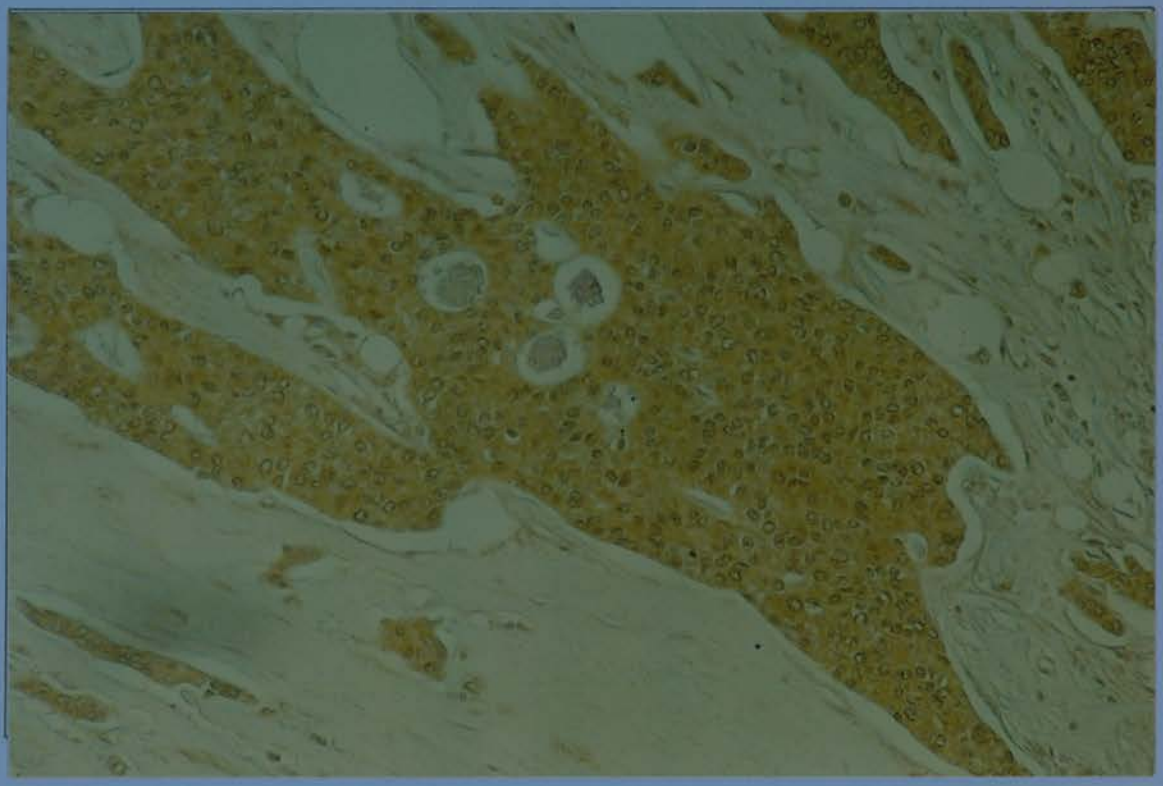


Table 28

Clinical features of the study population with uPA immunohistochemistry. Invasive cancer types are DCI = ductal carcinoma of no special type, LCI = lobular carcinoma, TCI = tubular carcinoma, and OTH = other special types

Table 28

	Number of cases	Percentage with positive immunohistochemistry
Invasive cancers	113	35
In situ cancers	21	19
Invasive cancers only		
Oestrogen receptor status		
< 20fmol/mg protein	32	41
> 20 fmol/mg protein	73	34
NK	8	
DNA ploidy		
Diploid	45	36
Aneuploid	28	43
Tetraploid	24	38
NK	6	
Cancer size		
1 - 10	10	50
11 - 20	38	24
21 - 30	41	44
31 - 40	12	33
> 40	12	33
Lymph node status		
positive	36	31
negative	68	38
NK	9	
Cancer grade		
1	21	24
2	66	38
3	21	38
NK	5	
Cancer Type		
DCI NST	86	35
LCI	14	50
TCI	10	20
OTH	3	33

X2 = 0.74, p=>0.10, <0.50. Grade I cancers showed a lower frequency of overexpression (24%) than grade II or III (38% each), but these differences were also not significant, X2= 1.12, p>0.5. Overexpression was not confined to any particular histological type of cancer, but noteworthy was the high proportion in lobular carcinomas (7 of 14), and overexpression was also present in some tubular carcinomas (2 of 10). Frequency of overexpression in each type of cancer is shown in Table 28.

Overexpression of uPA was significantly associated with overexpression of c-erb B2, X2 =6.47, p=0.01 , but not with c-erb B2 gene amplification, X2 =2.15, p=>0.10, <0.5, Table 29.

7.3.2 Validation of dPCR for Detection of uPA Gene Copy Number.

The relative efficiencies of primers sets for uPA and IFNG150 were tested in differential PCR. Optimal primer concentration was tested using 83, 125, 250 and 312pmol of urokinase primers with 250pmol of IFNG150 primers in a standard differential PCR on 2 normal fixed breast tissues and a placental control DNA. Differential PCR ratio values increased with increasing uPA primer concentration, Figure 53, and equimolar concentrations gave an approximate ratio value of 1 for each DNA. This concentration was used for all subsequent experiments.

A positive control was not available for uPA amplification, therefore a standard PCR reaction containing 200ng of placental DNA as template, was "spiked" with 1, 2, 4, 8, 16 and 32µl of uPA PCR product ($1x10^{-6}$ dilution) to provide an artificially increased uPA template. Measurement of the concentration of PCR product used for

"spiking" was attempted using standard spectrophotometry, fluorometry, and ethidium bromide staining methods.

Primer efficiency for uPA with IFNG150 was tested on control DNA and control DNA spiked with uPA product in a dPCR cycle course experiment, in which PCR product was sampled at 23, 25, 27, 29, 31 and 35 cycles. Ratio values tended to increase or decrease with increasing cycle numbers for the positive or negative controls respectively, Figure 54. This indicated some variation in primer efficiency but the differential between the two controls remained distinct.

The dPCR ratio values for positive "control" DNA in the standard dPCR for uPA are shown in Figure 55. Increasing quantities of "spiked" uPA product resulted in increased dPCR ratio values, indicating that the technique can detect increased uPA templates. Attempts to measure the concentration of uPA PCR product added to "spiked" reactions were unsuccessful due to the difficulty in measuring small DNA fragments in low concentration.

Table 29

Association between urokinase plasminogen activator (uPA) expression and c-erb B2 overexpression (A) and amplification (B).

A

c-erb B2 expression	uPA expression positive	negative	
positive	17	16	$X^2 = 6.51$
negative	27	74	$p = <0.01$

B

c-erb B2 amplification	uPA expression positive	negative	
positive	26	18	$X^2 = 2.15$
negative	42	47	$p < 0.10 > 0.05$

Figure 53

Titration of uPA primer concentration with 250pmol IFNG150 primers in a standard dPCR reaction. Differential PCR ratio values increase with increased uPA primer concentrations for normal breast tissue DNAs alh488n, alh490n and cell line DNA MCF7.

Figure 53 Titration of uPA primers in a standard dPCR reaction

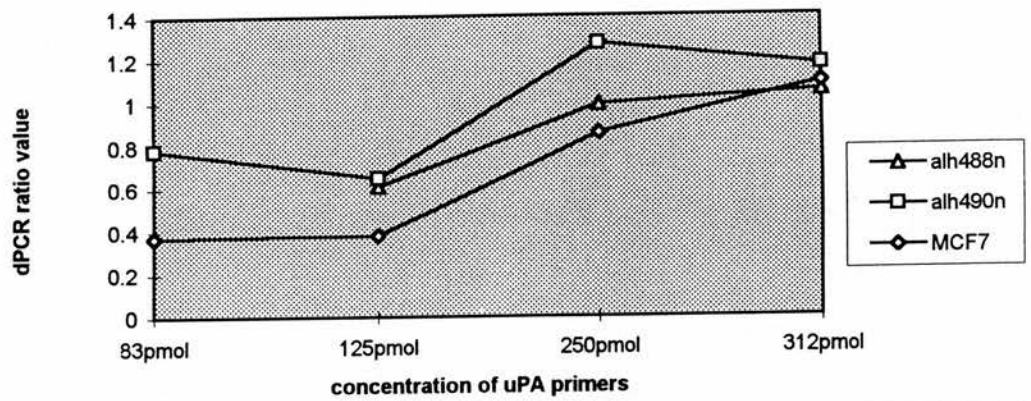


Figure 54

Differential PCR cycle course using equimolar concentrations of uPA and IFNG150 primers in a standard dPCR reaction. PCR product was sampled at cycle numbers 23, 25, 27, 30 and 35, and the results expressed as ratio values. Results indicate that the ratio value does not change with increasing cycle number for both the negative control (Sigma DNA) or the positive control.

Figure 55

Differential PCR ratio values obtained from control DNA (Sigma, UK Ltd) "spiked" with 1, 2, 4, 8, 16, and 32μl of urokinase PCR product to produce DNA templates which represent increases copy number of uPA gene. In a standard dPCR reaction ratio values increase with increasing uPA gene copy number.

Figure 54 Differential PCR ratio values for uPA throghout a thermal cycle course

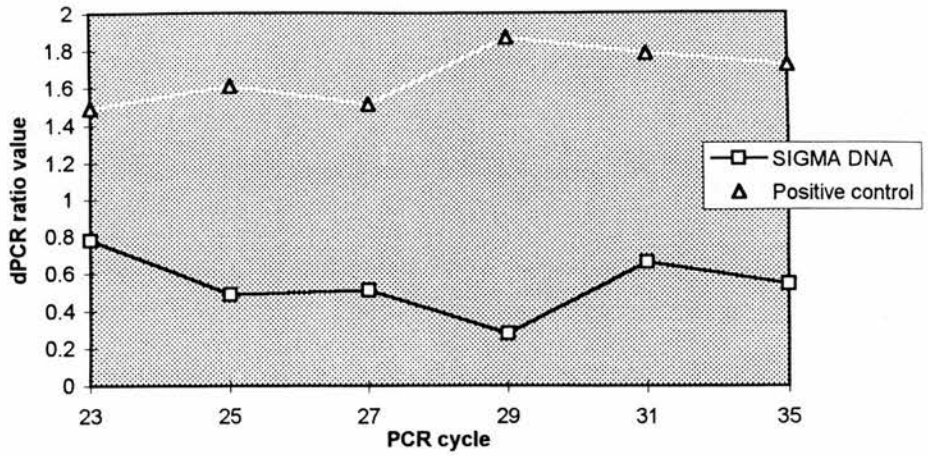
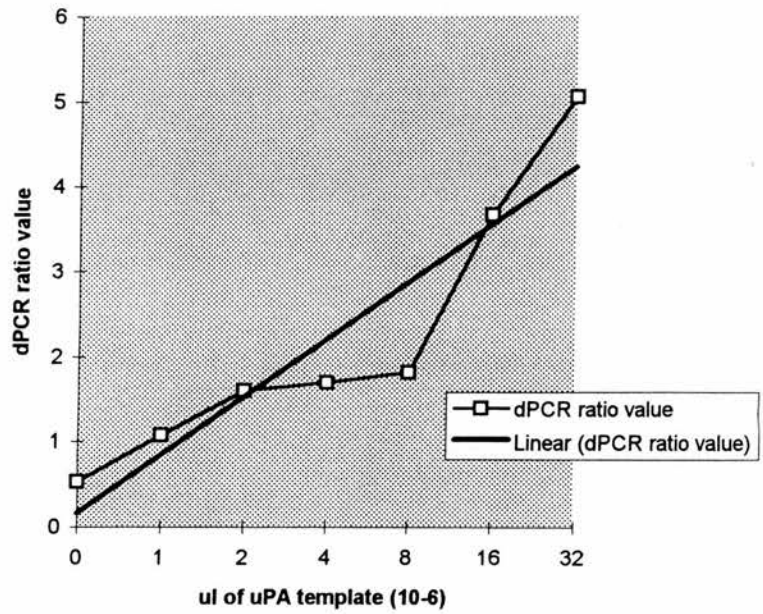


Figure 55 Effect on dPCR ratio values of additional uPA template with control DNA



7.3.3 Assessment of uPA Gene Copy Number in Breast Cancer.

Differential PCR ratio values were obtained for 134 cancer specimens and 33 control tissues, Figure 56, and range from 0.52 to 1.54 (mean 0.9) in control tissues and 0.41 to 1.83 (mean 1.1) in cancer tissues. Figure 57 shows representative dPCR products obtained from three cancer specimens and a normal DNA control (derived from human placenta). Ratio values of 2 or above have been considered to signify gene amplification with the oncogene c-erb B2, see Chapter 2. There was no clear difference in range of values between invasive cancers (0.41-1.83) and *in situ* cancers (0.7-1.55). The distribution of ratio values was similar for immunohistochemistry-positive and -negative cancers, Figure 58. Cancers shown to be overexpressing uPA protein are distributed evenly over the range of dPCR values, indicating that cancers which overexpress uPA are not associated with high dPCR values. Differential PCR values were not influenced by specimen cellularity or DNA ploidy.

Figure 56

Range of dPCR ratio values found for 134 breast cancers and 33 control tissues.

Figure 58

Range of dPCR ratio values obtained in a standard dPCR reaction for breast cancers which are either immunohistochemistry positive or negative cancers.

Figure 56 Distribution of dPCR ratio values for uPA in breast cancers and controls

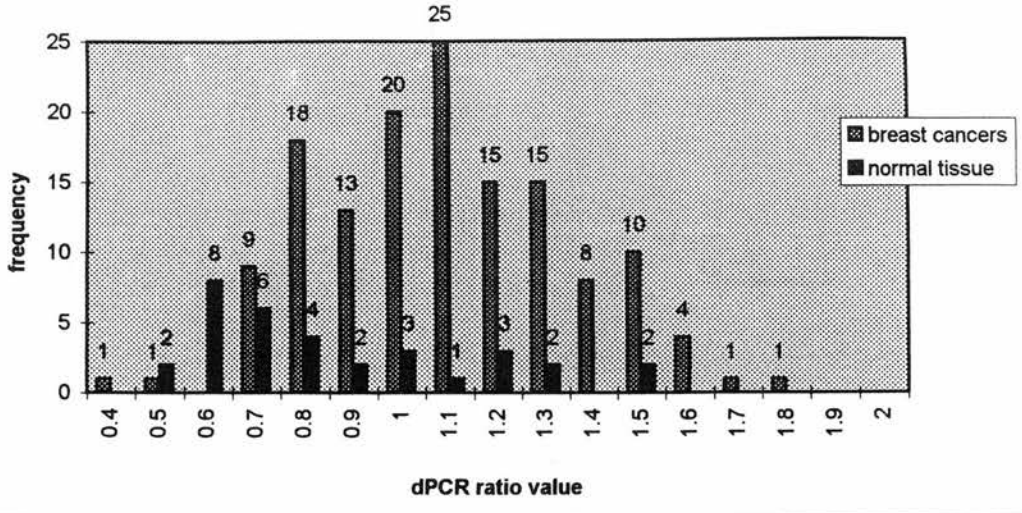


Figure 58 Distribution of uPA dPCR ratio values for uPA immunohistochemistry - negative and -positive cancers

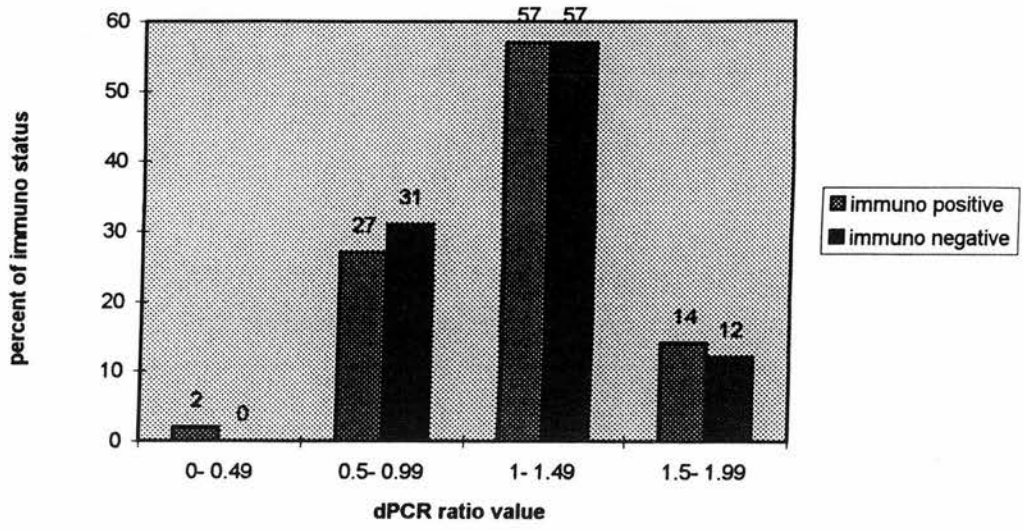
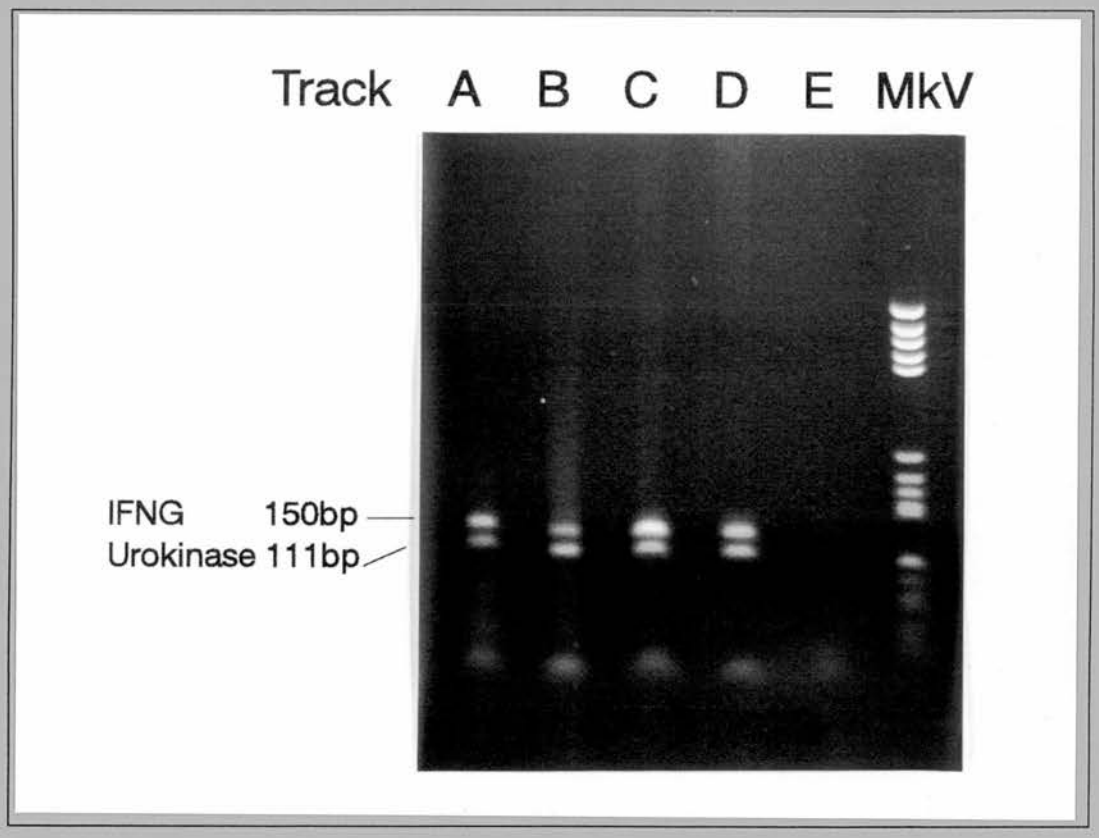


Figure 57

Differential PCR products from three different breast cancers (Tracks A and B and C) and one control placental DNA (Track D), separated on 2% 3:1 Nuseive/Seakem agarose gel. Track E contains the experimental negative control (PCR reaction minus template DNA). PCR products for IFNG (reference gene) and uPA are indicated at 150bp and 111bp respectively

Figure 57



7.4 DISCUSSION

The studies on uPA amplification presented above lead to two principle conclusions. First, the methods used - essentially the same as those devised for c-erb B2 in Chapter 2- produce a profoundly different result, for this unrelated gene. This gives further support for the power of this method in measuring gene amplification. Second, there was no evidence that uPA was amplified, and although immunohistochemistry showed overexpression in 33% of cases (including "early" ones), that was not related to uPA amplification

Differential PCR with primers for uPA and IFNG150 was validated using normal control DNA and DNA "spiked" with uPA product, and showed that, although there was some difference in primer efficiency, dPCR could detect uPA gene amplification if present in cancer DNA. This method of "spiking" control DNA with PCR product has been used to calibrate other dPCR studies (Lonn et al 1993), but its sensitivity in detecting gene copy number remains unproven as concentrations of small DNA fragments are difficult to measure accurately. Amounts of uPA added to "spiked" controls were selected for their similarity to those amounts of c-erb B2 PCR product required to "spike" normal control DNA to produce an amplified dPCR ratio value (data not shown), and suggests that uPA template added to dPCR was of the correct order.

Although no difference was detected between the ranges of dPCR ratio values for cancers and controls, cancers had a higher mean dPCR value. This could reflect a low level of chromosomal instability, yet there was no association between "high" dPCR values and abnormalities of DNA ploidy. Furthermore, cases overexpressing uPA protein were not solely restricted to those with dPCR ratio values at the upper

end of the normal range, indicating that overexpression was not due to minor chromosomal disregulation. The possibility that gene overexpression can lead to gene amplification has been suggested (Shen et al 1986, Kraus et al 1987) but has not been confirmed experimentally and seems unlikely to be true for the uPA gene.

The associations found between uPA and cancer aggression in some studies is commonly thought to be mediated by the breakdown of the extracellular matrix thereby facilitating cancer cell dissemination (Duffy et al 1990, Janicke et al 1990). I found uPA expression to be common in invasive lobular cancers. These cancers characteristically infiltrate through stromal elements as individual units in linear array, and uPA may play some part in this process. However, both invasive tubular carcinomas and *in situ* cancers also expressed uPA, yet these histological types are recognised for cell cohesion, differentiation and lack of metastasis (Bellamy et al 1993, Dixon et al 1985). This, and the presence of uPA in small, low grade, lymph node negative cancers suggests that overexpression of uPA can be an "early" event and that additional factors are required for the development of invasion resulting in a poorer prognosis. The presence of uPA in the cytoplasm is probably only one of several possible factors involved in cancer cell dissemination. Examination of lung carcinoma cell lines have indicated that functionally active, receptor bound, uPA is essential for tissue invasion (Bruckner at al 1992). Studies of uPA overexpression combined with assessment of cell surface receptors (uPAR) may illuminate the significance of overexpressing uPA, especially in cancers which have a favourable outcome.

Assessing the mechanisms accounting for overexpression of uPA in breast cancer is likely to be complex, and may involve factors which affect transcription, translation and posttranscriptional processing. Increased levels of specific uPA inhibitors, PAI-

1, PAI-2 and protease nexin, in breast cancer cells have been shown to regulate cytoplasmic concentration of uPA (Hart and Rehmtulla 1988). Post-transcriptional activation mechanisms of uPA may also be disregulated in cancer cells, resulting in an increase in stability and or processing of nuclear uPA transcripts (Henderson et al 1992). Immunohistochemistry is a convenient method for demonstrating disregulated gene expression and detection frequency is usually high, in the order of 88% to 100% of breast cancers (Janicke et al 1990, Sumiyoshi et al 1991). However, this study detected overexpression of uPA in only 33% of breast cancers, a frequency similar to another immunohistochemical study of breast cancer, although that study was based on frozen sections (Visscher et al 1990). This recorded disparity in frequency of positivity is a cause for concern, but could be due to criteria for scoring positivity, differences in immunohistochemical protocols and affinity of antibodies, or all of these. Present requirement for staining above that of normal stroma was an excluding factor in only 6 cases and is unlikely to be a major factor influencing the frequency of positivity. The instability of the antibody used here is a major factor causing concern and has been noted in other laboratories (M. Walch, personal communication) for unidentified reasons, this instability may reduce the sensitivity of detecting uPA protein. If the sensitivity is reduced then the uPA expression detected here may represent cancers with particularly high levels of uPA. There are alternative antibodies available for uPA which also detect high frequencies of expression in ELISA (Grondahl-Hansen et al 1993) but present experience suggests caution before drawing conclusions from immunohistochemistry alone.

Other features of our cancer population, such as size and ploidy, show no particular associations with uPA expression. The trends toward elevated uPA expression in grade II or III cancers and in negative lymph node status cancers observed above, were noted previously (Foekens et al 1992, Grondhal-Hansen et al 1993). However

in the majority of studies uPA expression did not vary according to classical prognostic factors, such as histopathological grade, lymph node status, oestrogen receptor status or cancer size, but remained significantly associated with shorter disease free and overall survival (Duffy et al 1990, Foekens et al 1992, Foucre et al 1991, Janicke et al 1990).

Correlations of biochemical and immunohistochemical procedures generally have indicated that measured concentrations of uPA protein correlate with the strength of immunostaining, although individual cases can show major discrepancies (Janicke et al 1990). This suggests that the relationship between immunohistochemical staining and detection of uPA protein in breast cancers is not strictly quantitative. Furthermore, the results of biochemical studies may be confounded by the presence of uPA-rich macrophages (Pyke et al 1993), indicating that a combination of immunohistochemistry and ELISA would be more informative than either technique alone. Comparability between studies is further confounded by differences in the experimental strategy and antibodies employed (Duffy et al 1988, Foekens et al 1992, Grondahl-Hansen et al 1993, Janicke et al 1990, Visscher et al 1990). The concentrations of uPA in the cytoplasm of cancer cells is known to be affected by growth factors, phorbol esters, cytokines and hormones (Butler et al 1979, Katzenellembogen et al 1984, Neidbala et al 1992, Rorth et al 1990, Stopelli et al, 1986). Studies of cell lines have shown that oestrogen complexed with its receptor can induce uPA synthesis (Katzenellenbogen et al 1984). I found no significant difference in the frequency of uPA protein overexpression in oestrogen receptor-positive and -negative cancers, suggesting that oestrogen is not a major influence in the complex regulatory mechanisms of uPA production.

uPA expression may also be associated with growth factor activity in breast cancer cells (Naldini et al 1992). Overexpression of c-erb B2, a member of the epidermal growth factor receptor family, has been observed in cancers which also overexpress uPA (Del Vecchio et al 1993). While that study examined only 22 breast cancers, a limited number of cases, my study confirms and extends this association. This may merely reflect the presence of two similar but unconnected markers of aggression in invasive cancer tissues, or it may reflect some biological linkage between these two genes. *In vitro* studies have shown that uPA expression can be increased by epidermal growth factor (EGF) (Neidbala et al 1990). C-erb B2 cannot bind EGF directly (Dougall et al 1994), but may be able to participate in the binding complex by forming a heterodimer with epidermal growth factor receptor (Dougall et al 1993). A direct effect of c-erb B2 activation could be mediated by an increase in cAMP activity (Dougall et al 1994) as uPA has an upstream sequence of DNA similar to corresponding regions of other cAMP regulated genes (von der Ahe et al 1988). Furthermore uPA protein has a partial amino acid sequence homology to EGF, in the region which binds to EGFR (Appella et al 1987) although the ability for uPA to bind to the receptor has not been shown. These possible interrelationships need to be clarified to evaluate biological relevance.

In summary, this study has provided evidence that uPA is overexpressed in at least some breast cancers which may relate to gene disregulation, though not to gene amplification. The c-erb B2 gene may be involved in some biochemical pathways for uPA expression.

Chapter 8

General Discussion

8.1 Introduction.

The role of the gene c-erb B2 in breast cancer has been under active investigation since the initial association of c-erb B2 gene amplification with aggressive behaviour in breast cancer (Slamon et al 1987). Several important questions remain and some were addressed in this thesis: what are possible causes or controls of disregulation of c-erb B2, how are the processes of disregulation interrelated, and at what stage(s) of cancer progression may c-erb B2 exert an influence?

I modified and extended the differential PCR technique for the detection of amplification of relevant genes, including c-erb B2, and uPA. This allowed an examination of the complex relationships between gene amplification, overexpression, and other features of breast cancers, and an examination of c-erb B2 polymorphisms in amplified cancers. Novel findings included aspects of disregulation of c-erb B2 in breast cancer and its possible effects on the biology of breast cancer. I will attempt to relate these findings to some general theories of breast cancer and genetic disregulation.

Current theories on breast cancer aetiology and progression are based mainly on morphological features of the disease, which suggest an initial *in situ* phase progressing to invasive forms (Cardiff 1988, Tubianna and Kocielny 1992), as discussed in Chapter 4. Numerous gene disregulations have now been identified in breast cancer but evaluation of their potential significance in disease progression is complicated by the lack of clear morphological markers of the stages in breast cancer. However, some familial cancers have now been shown to have disregulation of BRCA1 and BRCA2 (Wooster et al 1994, Miki et al 1994). While it is possible

that such genes may also be disregulated in sporadic breast cancers, it is likely that a more complex pattern of accumulated disregulations occurs in elderly women.

Other "candidate" genes for disregulation include tumour suppressor genes which may show a loss of function, or oncogenes which can gain function, with such disregulation, leading to neoplasia. C-erb B2 oncogene codes for a cell membrane receptor which shows close homology to the epidermal growth factor receptor. It can act as a dominant transforming gene in cultured mammary and other cells (DiFiore et al 1987, Pierce et al 1991), and in transgenic mouse mammary tissue (Muller et al 1988). Although c-erb B2 gene is frequently reported to be either amplified and/or overexpressed in human breast cancer tissue, its role in breast cancer initiation or progression is not yet clear.

8.2 Possible Mechanisms of c-erb B2 Gene Amplification.

Gene amplifications are thought to be rare in normal epithelial cells (Tlsty et al 1990), however transformed cells demonstrate greater genetic instability and can contain regions of DNA amplification involving several genes (Stark and Wahl 1984, Keith et al 1993). Genetic instability may occur in some cancers in a non-specific fashion, and include point mutations, deletions, translocations and amplification. Telomere shortening has been suggested as a cause of genetic instability (Shay et al 1993, Odagiri et al 1994). As the frequency of c-erb B2 amplification is thought to be the highest of the known oncogenes in breast cancer (Press 1990), it is possible that some specific mechanisms are also involved.

It has been suggested that increased gene expression may lead to amplification of that gene. For example, an *in vitro* study of the multiple drug resistance gene showed it was amplified after its expression was increased by treatment with cytotoxic drugs (Shen et al 1986). However, many other genes, such as urokinase plasminogen activator are known to become overexpressed but are not subsequently amplified (see Chapter 7). Nevertheless, this model was suggested for c-erb B2 gene amplification (Kraus et al 1987) based on the observations that overexpression was more frequent than amplification, and that amplification was usually accompanied by gene expression. As discussed above, the high frequency of c-erb B2 gene amplification, often without accompanying overexpression, in all types of cancers makes this mechanism only a circumstantial possibility, dependent on expression being a variable factor.

The exact sites of c-erb B2 amplification within the genome of affected cells is not known, therefore giving no clue as to possible mechanisms. It is known from *in situ* hybridization studies that c-erb B2 amplification occurs at multiple sites (Smith et al 1994), but differentiation between episomal and chromosomal locations was not possible. More information is known about other genes. Amplifications of n-myc gene in neuroblastomas are present as repeated amplicons within sections of DNA, known as homogeneously stained regions, on different chromosomes; with the retention of the single copy gene at the resident site (Amler et al 1992). Amplifications of multiple drug resistance gene are present as small circular DNA episomes, which amplify further to form visible structures, known as double minutes (Schoenlein et al 1992). These two possible amplification sites could be linked in some way, for example episomal DNA could be incorporated into the genome. Cytogenetic studies of breast cancer tissue have indicated the presence of homogenously stained regions but not double minutes; however analysis indicated

that c-erb B2 amplifications were not localised to the stained regions (Saint-Ruf et al 1990).

This study is the first to demonstrate that either one or both alleles of c-erb B2 can be amplified (Chapter 5). This suggests that amplification can arise by different mechanisms. The models of DNA amplification which involve replication of the affected gene (see Chapter 1.6.4) may be more likely to involve only one allele than both, assuming initiation of amplification is a random error within a chromosome. The models of DNA amplification which involve segregation of DNA fragments during cell division may be more likely to involve both alleles in the resulting amplicon. Interpretation of allele frequencies and genotype distribution is complicated by possible allele loss, which can occur at loci in the c-erb B2 region in over 50% of breast cancers, and may be more frequent in cancers with c-erb B2 amplification (Futreal et al 1992). It is therefore apparent that the mechanism of c-erb B2 amplification in breast cancer is not yet defined, although investigation of allele frequencies is a useful tool for further study.

It is thought that gene amplifications in sporadic cancers arise by chance over time, and are not repaired by normal DNA repair mechanisms. Once present, these amplifications could be selected for, in some manner, if the amplicon was functional and provided an indirect or direct cellular growth advantage. Both selection pressures and faulty DNA repair mechanisms may contribute to the incidence and maintainance of the high frequency of c-erb B2 gene amplification observed in this study. Disregulated DNA repair mechanisms might not be specific to c-erb B2 and could accompany multiple sites of DNA amplification. However, groups of cancers with c-erb B2 amplification do not appear to have higher frequencies of amplification of other oncogenes, located distant from c-erb B2. For example, analysis of breast

cancers for c-myc, c-erb B2 and int-2 and other amplifications found a very low rate of co-amplification of other oncogenes in c-erb B2 amplified cancers (Berns et al 1992, Zhou et al 1989). It is therefore possible that positive selection pressure exist for the presence of c-erb B2 amplification. These selection pressures could be indirect or direct.

Indirect selection pressure could be applied to a gene closely linked and co-amplified with c-erb B2. Breast cancers have been shown to harbour amplification units encompassing c-erb B2, THRA1, retinoic acid receptor and topoisomerase IIα (Keith et al 1993), with some expression of topoisomerase IIα. The latter three genes all have products known to be involved in gene transcription, signalling and repair respectively. They could hence provide an indirect selection pressure if present as functional amplicons.

Examples of this kind of indirect selection pressure do exist for other oncogenes. The oncogene int-2 is amplified in some breast cancers (Tsuda et al 1989) and the int-2 amplification unit on chromosome 11q13 usually includes other genes, such as bcl-2, which can also be amplified independently of int-2 (Theillet et al 1990, Saint-Ruf et al 1991a). These other genes in the int-2 region are thought to be of significance in cancer progression, with the int-2 gene of lesser direct relevance (Brada 1992).

A direct selection pressure for the presence of c-erb B2 amplification could involve the expression of its growth factor receptor product, giving a direct advantage to cell growth after expression from a functional amplicon. The overexpression of c-erb B2 protein in breast tissue has been associated with an increased rate of proliferation of the cancerous breast epithelial cells, as measured by thymidine incorporation (Barnes

et al 1991) or S phase fractionation (O'Reilly et al 1991). This association was strong in *in situ* cancers, particularly of the comedo type, but only weak in invasive cancers (Barnes et al 1991, O'Reilly et al 1991). Taken as a whole, this data could indicate that the c-erb B2 protein can directly influence cancer cell growth, possibly providing a positive selection derived from the amplification. However there is a long chain of events implicit in this concept, with numerous likely qualifications, of which little is known.

Functional oncogene amplifications are only thought to occur in tissues where these genes are expressed at some point during normal development (DePinho et al 1991). C-erb B2 protein is thought to be present in low concentrations on the membrane of normal adult breast epithelial cells and is also expressed in the embryo during its normal development (Dougall et al 1994). It may have a role in normal cell differentiation and morphogenesis (Dougall et al 1994). The possible relationships between amplification, functional expression and oncogenesis may therefore occur over a long period.

In this study, c-erb B2 gene amplification was a common event and it was clearly detected in some cancers without an accompanying gene product overexpression. In a few cancers, gene overexpression occurred in the absence of amplification. Other studies have suggested that c-erb B2 amplification is not a frequent event (20 to 25% of breast cancers) and is nearly always accompanied by overexpression (Walker et al 1989b, Uehara et al 1990). It is therefore possible that my techniques used to measure amplification are more sensitive than previous studies. The considerable effort validating the parameters of my techniques indicated that the results are broadly correct, indicating that c-erb B2 disregulation may be more common than

previously recognised. Similarly, the use of new monoclonal antibodies (not used in this study) has led to an increase in detection of cancers overexpressing c-erb B2 gene product (Poller et al 1992). The study of the screened cancers suggested that c-erb B2 amplification occurred in all cancer histopathological types, including those with "early" features. Taken together, these indications that c-erb B2 amplification is an early and common event suggest that the gene may either be involved early in the oncogenic process, or that it is a secondary event conferring a growth advantage. Recent *in situ* hybridisation studies have indicated that transcriptional regulation may influence the relationship between c-erb B2 amplification and its expression in cells of an individual cancer (Smith et al 1994). The finding of a few cancers with overexpression, but no amplification in this study, also suggests that cellular mechanisms for increasing expression may operate alone. It is possible that these mechanisms could also operate in amplified cancers but this speculation has not been tested.

8.3 Expression of c-erb B2.

Although gene amplification is an important marker of gene disregulation, gene expression is more likely to exert a biological effect on the cancer cell. There may be an important role for mechanisms which control gene transcription, including the action of factors called promoters, enhancers or suppressors. These usually bind to a sequence of DNA upstream from the relevant gene. This upstream sequence is designated the promoter region, and both promoters and suppressors usually bind within this region. Other factors known as enhancers may also bind within gene introns, but few relevant studies are available.

The promoter region of c-erb B2 gene may contain sites for multiple mechanisms of transcriptional initiation (Tal et al 1987). However, few promoter factor molecules specific for c-erb B2 have been identified. Cell derived DNA binding proteins OB2-1 and HPBF have been shown *in vitro* to bind specifically to c-erb B2 promoter region, resulting in an upregulation of mitogenesis and c-erb B2 expression (Hollywood and Hurst 1993, Sarkar et al 1994). These promoters may themselves be subject to disregulation. Indeed HPBF was only detectable in cytoplasmic extracts from malignant cells (Sarkar et al 1994).

More studies have examined the role of suppressors, due to the recognition that oestrogen receptor may be involved in this type of action in breast cancers. Oestrogen receptor is thought to be involved in the transcriptional control of c-erb B2 gene (Russell and Hung 1992, Antoniotti et al 1994), but specific response elements (receptor binding sequences) have not been identified within the promoter region of c-erb B2. A particularly strong association between oestrogen receptor status and c-erb B2 expression was observed in this study set (Chapter 4), where the absence of oestrogen receptor correlated with the overexpression of c-erb B2 in nearly all cases, as found in other recent studies of similar study sets (Schroeter et al 1992, Schonborn et al 1994). This concurs with other *in vitro* studies, which have found that breast cancer cells containing oestrogen receptors stimulated by oestradiol also had a marked downregulation of c-erb B2 expression (Russell and Hung 1992, Antoniotti et al 1994). The mode of action of oestrogen receptor on the c-erb B2 gene is not known, but may include an intermediary step between the two, possibly involving c-myc (Suen and Hung 1990).

This strong association of oestrogen receptor with c-erb B2 expression has great relevance to analysis of the clinical features of breast cancers. The treatment of

cancers with the anti-oestrogen tamoxifen, may act to downregulate c-erb B2 expression (LeRoy et al 1991). However, addition of tamoxifen to breast cancer cells caused an increase in the c-erb B2 expression (Antoniotti et al 1992), so the association between oestrogen receptor, tamoxifen and c-erb B2 may be complex.

The nuclear receptor gene THRA1, which shares considerable homology to oestrogen receptor (King 1992) was also examined for possible involvement in c-erb B2 suppressor activity (Chapter 6), but none was found. THRA1 and c-erb B2 genes are co-amplified in approximately 20% of breast cancers (Tsuda et al 1989, Tavassoli et al 1989), but this may be merely due to physical linkage on chromosome 17q21. While my attempts to determine the co-amplification of THRA1 were not fully successful, SSCP analysis of the zinc finger DNA sequences showed that the binding specificity of the receptor molecule remained unaltered.

8.4 Cancer Progression and c-erb B2

I have discussed the possible role of c-erb B2 in cancer cell proliferation. However, there are other elements in cancer progression, such as metastatic potential. C-erb B2 overexpression has been suggested to be more common with node positive cancers (Berger et al 1988, Borg et al 1990) and c-erb B2 expression has been linked to the intercellular adhesion molecule, ICAM-1 and to urokinase plasminogen activator, uPA, both of which are thought to be involved in cancer cell dissemination.

Unlike some previous studies, my estimations of c-erb B2 disregulation, either amplification or overexpression, found no relationship to node positive status (Chapter 4). This does concur with some other similar studies (Gusterson et al 1988, Thor et al 1989). The reason for these apparent discrepancies is not clear, but may

reflect selection of cases for study sets. My study set drew from a wide screening population so may be more representative of a longer progression period. The intercellular adhesion molecule ICAM1 aids tissue cohesion, by binding cells to each other. In one study, it was found to be expressed in invasive breast cancers which were also positive for c-erb B2 protein overexpression (Bacus et al 1993). However, most node negative invasive cancers in that study were negative for ICAM1 expression, indicating a possible role in metastasis.

The urokinase plasminogen activator uPA can act to break down the extracellular matrix of solid tissue, thereby promoting cancer cell dissemination. It has been shown to be overexpressed in epithelial cells in some invasive breast cancers (Chapter 7, Foekens et al 1992, Grondahl-Hansen et al 1993). I demonstrated that overexpression of uPA was not due to gene amplification, therefore transcriptional control mechanisms must play an important role. Most cancers which overexpressed uPA also overexpressed c-erb B2 protein (Chapter 7), suggesting either a possible link, such as a common mechanism of transcriptional control, or coincidence. The many factors known to affect the expression of uPA (Hart and Rehemtulla 1988) would complicate any detailed analysis.

Although the biological functions of c-erb B2 remain unresolved, it is clear that there is a high frequency of disregulation at this locus. This could indicate that it may play some role in cancer progression. Establishing a role for candidate genes in cancer progression has proved difficult due to the considerable heterogeneity within the disease. I have been fortunate to be able to study cancers which may represent different chronological stages of the disease, namely prevalence, incidence, interval and never screened cancers (Chapter 4), and thence relate the molecular disregulation of c-erb B2 to these sets.

The newly screened group (prevalence cancers) contained more cancers with "early" characteristics, such as *in situ* and special type cancers, and small, node negative, oestrogen receptor positive cancers. The never screened group contained more cancers with "late" characteristics, such as invasive carcinomas of no special type, and large, node positive, oestrogen receptor negative cancers. Interestingly, the previously screened group (incidence and interval cancers) contained a number of cancers (approximately 40%) with "late" characteristics, particularly high grade invasive cancers and oestrogen receptor negative cancers. A proportion of cancers in the newly screened group will be slow growing and may be at a screen detectable stage over a long time period, therefore the prevalence screen will detect many more small, low grade cancers. Less of these cancers will be detected on incidence screening because of the relatively short time period between screens. This may indicate that a "length bias", could occur in the prevalence screen.

Cancer size and lymph node status are probably the most reliable characteristics of "early" or "late" cancer status, but histopathological type and grade, oestrogen receptor status and DNA ploidy are also of value for interpretation of cancer progression, see Table 20 Chapter 4. An important finding was that c-erb B2 gene amplification was not significantly associated with any of these cancer characteristics (Chapter 4). It would therefore appear to be an independent but common event, suggesting that it could be an early and possibly stable disregulation in some cancers.

8.5 Hypothesis and Model.

In contrast to c-erb B2 gene amplification, c-erb B2 gene product overexpression was significantly associated with histopathological type and oestrogen receptor status

(Chapter 4). A model of breast cancer progression, incorporating a previous model (Allred et al 1992) and results of this study is illustrated in Figure 59. It is suggested that overexpression decreases within individual tumours as they evolve from *in situ* to increasingly invasive lesions. Alternatively, invasive carcinomas, negative for c-erb B2 product overexpression, could arise *de novo* without progressing through a significant *in situ* stage.

It is possible that c-erb B2 protein overexpression is an early event in cancer initiation, rather than in cancer progression, as overexpression is common in "early" *in situ* cancers (Allred et al 1992, Chapter 4). In this study, c-erb B2 amplification was found to be an early event, but product overexpression was associated with both "early", *in situ* and "late", high grade, oestrogen receptor negative cancers. The latter finding could be explained by the strong relationship between c-erb B2 product overexpression and absence of oestrogen receptors, see Figure 60. The findings of this study therefore suggest that c-erb B2 amplification and product overexpression are early events, but that overexpression may not be a stable event, as it can be affected by other changes in cellular biochemistry.

Studies of gut and nervous system cancers have suggested that amplification of c-erb B2 and other genes is a relatively late event in progression of these cancers (Brodeur et al 1984, Ekstrand et al 1991, Ranzani et al 1990). Previous determination of the chronology of genetic events in breast cancer is complicated because results are often derived from symptomatically detected cancers, which may represent a relatively "late" stage of progression. In this study, cancers of likely "early" status still had a high rate of c-erb B2 amplification and amplifications were just as frequent in large cancers, as is suggested by some other studies (Iglehart et al 1990, Borg et al 1990). The amplification of c-erb B2 may therefore occur differently to that of oncogenes in

Figure 59.

An illustration of a proposed model of breast cancer progression, with suggested levels of c-erb B2 product overexpression in the various cancer types.

Level of c-erb B2 product overexpression is represented by:

*** High, > 50% of cancer group;
** Medium, 15 to 50 % of cancer group;
* Low, 0 to 15 % of cancer group;
a C-erb B2 product not detected;
b Not known.

DCI NST = invasive ductal carcinoma, no special type.
Histopathological grades I, II and III are described in Chapter 4.

Figure 59

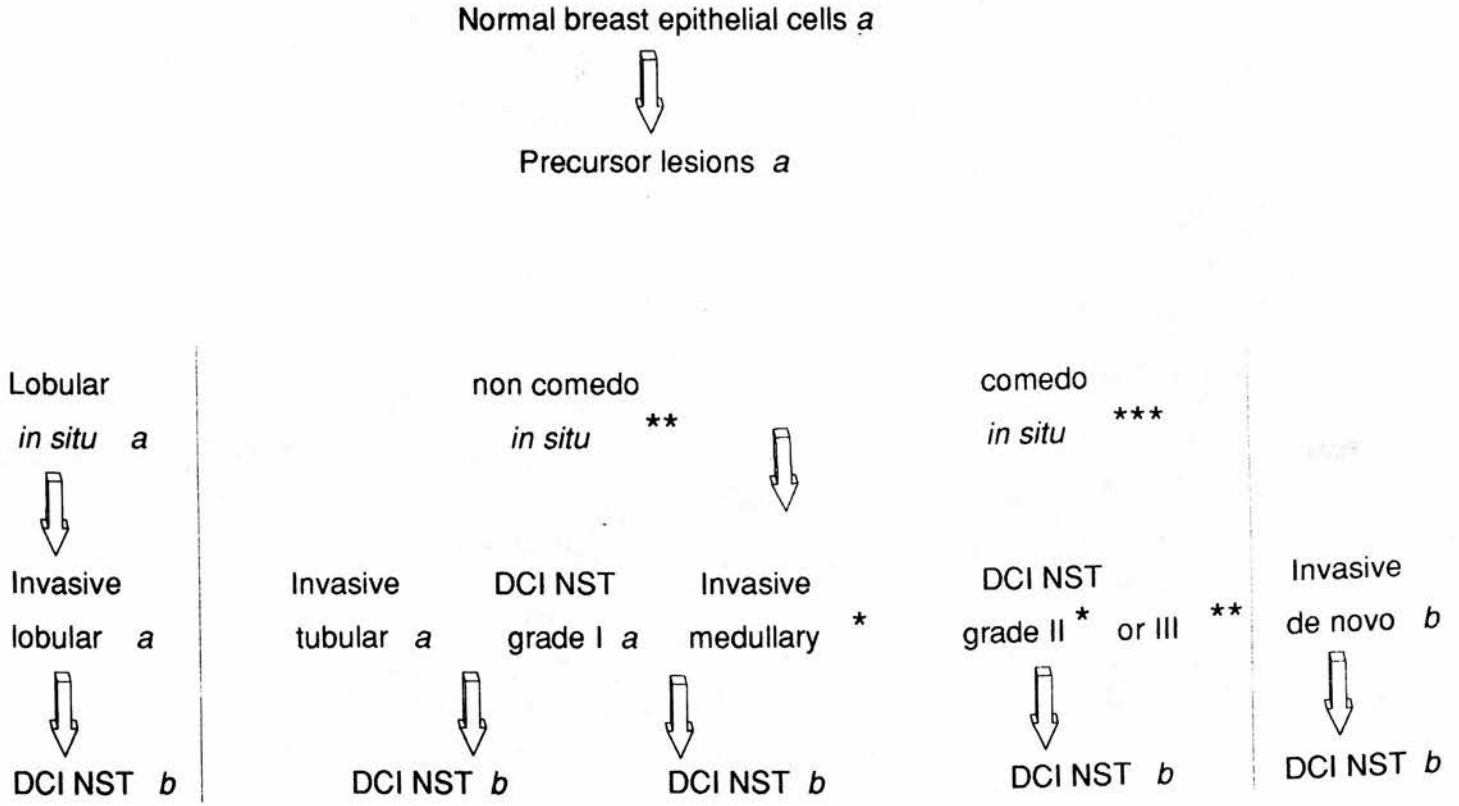
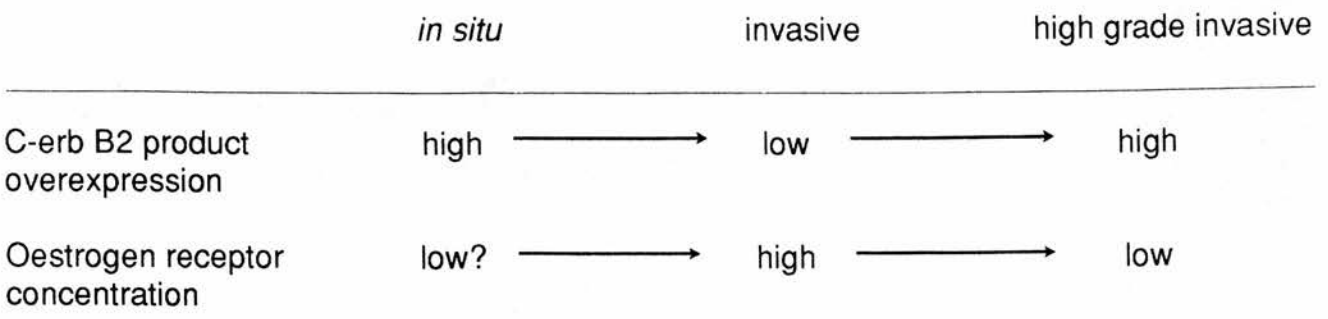

Normal breast epithelial cells a
Precursor lesions a
Lobular
in situ a
Invasive
lobular a
DCI NST b
non comedo
in situ **
Invasive
tubular a
DCI NST
grade I a
DCI NST b
Invasive
medullary *
DCI NST b
comedo
in situ ***
DCI NST
grade II * or III **
DCI NST b
Invasive
de novo b
DCI NST b

Figure 60.

A model of the relationship between c-erb B2 product overexpression and oestrogen receptor status in *in situ* and invasive cancers.

Figure 60


in situ
invasive
high grade invasive
C-erb B2 product overexpression
high
low
high
Oestrogen receptor concentration
low?
high
low

other groups of cancers. Understanding the mechanisms of its amplification could help to determine the role of c-erb B2 in breast cancer progression. The finding of disregulated c-erb B2 in "early" breast cancers, has not been reflected in any of the proposed precursor lesions, such atypical hyperplasias (Allred et al 1992), but these were not examined in this study.

8.6 Future Developments

This study has highlighted the need for a fuller understanding of the biology of c-erb B2 in breast cancer in order to assess its relevance to the disease. Initiation and progression represent different stages of cancer development and my study only addressed the latter. The complex factors which control the expression of c-erb B2, and the uncertainty about its biological functions make it possible that associations of c-erb B2 disregulation with clinical features of cancers may be subject to unseen bias. The presence of normal or disregulated c-erb B2 may therefore have some unseen beneficial function. Interestingly, some therapies of breast cancer have been designed to reduce the activity of the c-erb B2 gene, including anti-p185 monoclonal antibodies (Hudziak et al 1989) or oligonucleotides capable of binding to c-erb B2 mRNA (Colomer et al 1994). While my study of c-erb B2 in breast cancer has made a significant contribution to an understanding of its role, many other aspects of breast cancer remain to be explored. The availability of screen detected cancers gives a valuable study set for continued work. Areas of interest in future studies could include an examination of the mechanisms controlling transcription of c-erb B2, such as the nature of the promoter region; an analysis of c-erb B2 disregulation in familial cancers; and an analysis of c-erb B2 disregulation in incidence and interval cancers for better chronological separation of cancer status.

Appendix 1

General Solutions and Reagents

1.	Tris Borate buffer (TBE)	0.5x	0.045 M Tris-borate 0.001 M EDTA
2.	Tris EDTA buffer (TE)	1x	100mM Tris HCl to pH 8.4 2mM EDTA
3.	Tris buffered saline	1x	25mM Tris HCl, pH 7.4 5mM KCl 137mM NaCl 0.01% phenol red
4.	Ethidium bromide	stock	10mg/ml dH_2O
5.	Ammonium persulphate	stock	10% w/v in dH_2O
6.	Isopropylthio-β-D-galactoside (IPTG)	stock	20% w/v in dH_2O
7.	Chloroform:isoamylalcohol		24:1 v/v
8.	5-Bromo-4-chloro-3-indolyl-β-D-galactoside (x-gal)		20mg/ml dimethylformamide
9.	Proteinase K	stock	20mg/ml in H_2O
10.	Agarose gel loading buffer	10x	30% w/v Ficoll 0.25% w/v Orange G (Sigma) 0.5mM EDTA
11.	Sequencing gel loading buffer	2x	98% deionised formamide 50mM Tris borate pH8.3 10mM EDTA 0.025% w/v xylene cyanol FF 0.025% w/v bromophenol blue

12. N,N,N',N'- Tetramethyl-ethylenediamine (TEMED) Sigma Ltd 99.7% pure.

13. Lysis solution for preparation of DNA from fixed paraffin embedded tissues.

50mM Tris pH 8.4
1mM EDTA
0.5% Tween 20

14. L-Broth

10g/L Bacto-tryptone
10g/L NaCl
5g/L Yeast extract

15 L-amp plates

as L-Broth with 12g/L Bacto agarose
10μg/ml ampicillin (added when the agarose has cooled to less than 60°C).

16. SOC Medium

2% bactotryptone
0.5% yeast extract
10mM NaCl
2.5mM $MgCl_2$
10mM $MgSO_4$
20mM glucose

Appendix 2

General Methods

1. DNA extraction from frozen tissue.

1. Chop frozen tissue into small pieces in 0.5ml Tris EDTA, 10% SDS.
2. Add proteinase K to 0.5mg/ml and incubate samples overnight at 48°C, mix samples and return to 48°C for a further 24hours.
3. Follow protocol for purification and precipitation of DNA (see below).

2. Purification of nucleic acids.

Extraction with phenol:chloroform

1. Add an equal volume of Tris saturated phenol to DNA sample and mix till an emulsion forms.
2. Centrifuge the mixture at 12,000g for 1 minute at room temperature.
3. Remove aqueous phase to fresh tube.
4. Repeat steps 1-3 till no protein is visible at the interface.
5. Add an equal volume of 24:1 chloroform:isoamylalcohol and repeat steps 2-4.
6. Recover DNA by precipitation with ethanol

3. Precipitation of DNA

1. Add 1/10th volume of 0.3M sodium acetate (pH5.2), and 2 volumes of ethanol to DNA in solution, mix by inversion.
2. Cool at -70°C for 30 minutes
3. Centrifuge at 12,000g for 10 minutes.
4. Remove supernatant.
5. Wash DNA pellet in two volumes of 70% ethanol.
6. Centrifuge at 12,000g for 10 minutes.
7. Remove supernatant
8. Repeat steps 5-7 once.
9. Dry DNA pellet under vacuum.
9. Resuspend DNA pellet in Tris EDTA pH8.4.

4. Dynabead purification and preparation of single stranded biotinylated PCR product (Dynal)

1. Prepare Dynabeads. Throughout, superparamagnetic Dynabeads are separated from supernatant fluids using a magnetised strip. For each DNA sample wash 20µl of Dynabeads twice in 20µl of DNA binding and washing solution (Dynal, 2x = 10mM Tris HCl {pH 7.5}, 1mM EDTA, 2M NaCl). The final pellet of beads is resuspended in 40µl of binding and wash solution.

2. Add 40µl of amplified DNA to 40µl of washed Dynabeads, and incubate for 15 minutes at room temperature, mixing gently. Magnetise beads and remove supernatant.

3. Wash beads twice in binding and washing buffer as in step 1.

4. Resuspend beads in 8µl of 0.1M NaOH, and incubate at room temperature for 10 minutes. Separate beads and remove supernatant to fresh tube (contains non-biotinylated DNA strand).

5. Repeat step 4.

6. Wash beads once in 40µl of binding and wash buffer, and once in 50µl TE buffer. Resuspend final bead pellet in 20µl distilled water.

5. Wizard ^{TM}Miniprep DNA purification system (Promega).

Preparation of DNA from plasmid DNA from *E.coli.* cultures.

1. Pellet 3ml of bacterial cells by centrifugation for 2 minutes at 12,000g. Resuspend cell pellet in 200µl of Cell resuspension solution (Promega) and add 200µl of Cell lysis buffer (Promega), mix by inversion. When solution clears add 200.µl of Neutralisation solution (Promega). Centrifuge at 12,000g for 5 minutes. Decant supernatant to new microcentrifuge tube.

2. Add 1ml of Wizard Resin to the supernatant from step 1. Push resin/DNA mix into mini column (Promega) using a 3ml disposable syringe. Push 2ml of Column wash solution (Promega) through resin in mini column.

3. Centrifuge mini column at 12,000g for 20 seconds. Add 20.µl of distilled water to top of mini column, and incubate at 65^{o}C for 1 minute. Centrifuge at 12,000g for 20 seconds. The supernatant contains purified plasmid DNA.

6. Purification of DNA using G50 sephadex columns.

Quick Spin™ Columns (Boeringer Mannheim Biochemica Ltd) contain 0.8 ml of pre-swollen Sephadex G-50 in 10mmol Tris HCl pH8, 1mmol EDTA, 0.1mol/l NaCl.

1. Mix sephadex by inversion to resuspend the medium within the column, and centrifuge at 1100 rpm for 2 minutes. Discard eluted buffer.

2. Add DNA solution to the top of the sephadex column. Centrifuge for 4 minutes at 1100 rpm. The eluate from this spin contains the purified DNA sample.

7. Nuseive:Seakem (FMC Ltd) agarose gels

General agarose gel for separation of PCR products.

1.5% w/v Nuseive agarose
0.5% w/v Seakem agarose
in 0.5x TBE

1. Heat in microwave to melt agarose, cool to 55°C. Add 1/10,000th volume of ethidium bromide. Pour gel into gel cast, allow to set at 4°C.

8. Metaphor agarose gels

1. Add 1.8% w/v metaphor agarose (FMC Ltd) to 0.5x TBE buffer.
2. Heat in microwave till agarose dissolves.
3. Cool to <55°C, while stirring
4. Add 1/10,000th volume of Ethidium bromide
5. Pour gel at room temperature.
6. When set, cool at 4°C for 30 minutes prior to electrophoresis.

9. MDE™ Polyacrylamide Gel

0.5x MDE gel	25ml MDE gel.
	69ml deionised water
	6ml 10x TBE buffer
	0.4ml 10% ammonium persulphate
	0.04ml TEMED

10. Denaturing polyacrylamide gel

5% polyacrylamide gel used to separate DNA sequencing reactions.

For 50ml of gel

5ml 10x TBE buffer
8.33ml 40% (29:1) Acrylamide:bis acrylamide
40% Urea
36.67ml dH_2O
25µl TEMED
250µl 10% ammonium persulphide

After pouring allow to set at room temperature for 1 hour. Preheat gel to 55°C prior to electrophoresis.

11. Silver staining polyacrylamide gels

Biorad kit - Silver Stain Plus no 161-0449

1. Immerse polyacrylamide gel in 400mls of gel fixative, with gentle shaking for 20 minutes.

Gel fixative	200ml Methanol
	40ml Acetic Acid
	40ml Fixative enhancer concentrate (BioRad)
	120ml Deionised Distilled Water.

2. Pour off fixative and rinse gel twice in 400mls deionised distilled water for 20 minutes.

3. Pour off water and immerse gel in 300mls staining solution, with gentle shaking for approximately 20 minutes, or until desired staining intensity is reached.

Gel stain	15mls Silver Complex Solution (BioRad)
	15mls Reduction Moderator Solution (BioRad)
	15mls Image Development Reagent (BioRad)
	105mls Deionised Distilled Water
	150mls Development Accelerator Solution (BioRad)

4. Pour off developer solution and stop reaction in 5% v/v acetic acid in deionised distilled water for 15 minutes with gentle shaking

5. Rinse gel in 400mls of deionised distilled water.

6. Dry gel on filter paper support on Hoeffer vacuum gel drier for 2 hours.

12. DNA Sequencing

A. Cyclist™ sequencing kit (Stratagene)

1. Add 3µl of the appropriate ddNTP to each of 4 termination tubes. Cap and keep on ice.

2. For each DNA template to be sequenced the following reagents were mixed

Template 500fmol
Primer (1pmol)
4µl of 10x sequencing buffer
10µCi α-^{35}S dATP
2 units Taq polymerase
water to final volume of 30 µl.

3. Aliquot 7µl of the reaction mixture from step 2 into each of the 4 termination tubes from step 1.

4. Overlay with 15µl of mineral oil.

5. Cycle the sequencing reaction through an appropriate temperature profile.

Denature at 95°C for 5 minutes, then 30 cycles of
95°C for 30 seconds
55°C for 30 seconds
72°C for 60 seconds

6. Add 5µl of stop mix to the reaction.

7. Heat denature at 80°C for 5 minutes, then load 3µl of each sample onto a sequencing gel.

B. Sequenase T7 DNA polymerase (United States Biochemical)

1. Annealing reaction. For each template DNA a single annealing reaction is mixed.

0.5pmol Primer
1pmol Template DNA
2µl Annealing buffer (USB)
H_2O to 10ul

Warm annealing mixture to 65°C for 2 minutes, allow to cool to room temperature over 30 minutes. Chill on ice

2. Labelling reaction. To the annealing mix add the following;
   1µl DTT (0.1M)
   2µl Labelling nucleotide mix
   5µCi α-^{35}S dATP
   3 units Sequenase enzyme

Incubate at room temperature for 3 minutes.

3. Termination reaction. Add 2.5 µl of the appropriate ddNTP to each of four termination tubes. Prewarm to37°C

Add 3.5µl of the labelling reaction to each of four ddNTP tubes. Incubate at 37°C for 5 minutes. Add 4µl of stop solution to each tube.

Heat denature samples at 95°C for 5 minutes prior to loading 3µl per lane on a polyacrylamide gel.

13. Fresh tissue fixation and processing.

Fresh breast tissue was fixed in 6:3:1 methanol:chloroform:acetic acid at 4°C overnight. Fixed tissues were processed to paraffin wax using the following protocol.

1.	70% alcohol	1 hour
2.	64op alcohol	1.5 hours
3.	64op alcohol	2 hours
4.	74op alcohol	2 hours
5.	74op alcohol	2 hours
6.	absolute alcohol	2 hours
7.	absolute alcohol	2 hours
8.	Xylene	2 hours
9.	Xylene	2 hours
10.	Xylene	2 hours
11.	Wax (Ralwax1)	2 hours
12.	Wax (Ralwax1)	2 hours
13.	Vacuum in wax bath in processing room.	
14.	Embed in routine wax (Miles)	

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1. A.L. Hubbard, T.J. Anderson. (1993) Simple 10 minute preparation of fixed, embedded breast tissue for the polymerase chain reaction. *The Breast* **2** 50-51.

2. A.L. Hubbard, C.P. Doris, A.M. Thompson, U. Chetty, T.J.Anderson. (1994) Critical determination of the frequency of c-erb B2 amplification in breast cancer. *British Journal of Cancer* **70** 434-439.

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Minor publications

1. A.L. Hubbard, T.J. Anderson. (1992) Assessment of c-erb B2 amplification in breast cancer by the differential PCR method. *The Breast* **1** 164.

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Critical determination of the frequency of c-*erb*B-2 amplification in breast cancer

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Summary Tissues from 323 methacarn-fixed and paraffin-embedded breast cancers were assessed for c-*erb*B-2 gene amplification by differential polymerase chain reaction (dPCR). The sensitivity of dPCR was ascertained using cell lines with c-*erb*B-2 amplification, and the relationship between dPCR ratio value and gene copy number was established. In clinical material the technique was not affected by the DNA contribution of normal tissue elements or by cancer DNA ploidy change. c-*erb*B-2 gene amplification was detected in 55% of invasive cancers and in 66% of *in situ* cancers. c-*erb*B-2 protein overexpression in breast cancer cells, as determined by specific immunohistochemistry, was only detected in 11% of invasive cancers and 43% of *in situ* cancers. Comparisons show that a substantial number of cancers with c-*erb*B-2 amplification lack detectable protein overexpression. This illustrates the complex nature of c-*erb*B-2 gene disregulation in cancer and suggests that multiple combinations of biological events and consequences are possible.

Disregulation of the proto-oncogene c-*erb*B-2 (also known as HER-2/*neu*) has been implicated in the aetiology of breast cancer. Since the publication of a study linking c-*erb*B-2 to poor prognosis in breast cancer patients (Slamon *et al.*, 1987) there have been many studies examining c-*erb*B-2 gene amplification, mRNA production and protein overexpression. Recent reviews have collated the results from over 50 studies and found a general agreement between them on the frequency of c-*erb*B-2 disregulation in terms of gene amplification and protein overexpression, as measured by Southern blotting and immunohistochemistry respectively (Perren, 1991; Singleton & Strickler, 1992). However, there are major differences in the association of c-*erb*B-2 disregulation with histopathological features and with prognosis, making its involvement in cancer development and progression difficult to determine. It is not clear whether differences in results between studies have been the result of variations in sample selection, experimental technique or genuine biologically relevant disparity between populations.

Of the techniques for measuring gene amplification, Southern or dot blotting suffers from the disadvantages that microgram quantities of DNA are required for analysis and tissue morphology is destroyed in the extraction process. Recent advances in polymerase chain reaction (PCR) technology have made possible the analysis of minute quantities of DNA, with semiquantitative differential estimations (dPCR) demonstrating increased gene copy number in cell lines (Frye *et al.*, 1989). The present work explores the sensitivity of dPCR in detecting an increased gene copy number in a large series of clinical cancers by extending the application of this technique to paraffin-embedded tissues, with a view to evaluating the relationship between c-*erb*B-2 gene amplification and expression.

Materials and methods

Study set

The study tissues (336 cases) were collected from primary operable (clinical stage I and II) breast cancers at routine operations, which included mastectomy and excisional biopsy for both palpable and non-palpable lesions. Samples were restricted to the age group 50–65 and were collected over the period of January 1988 to May 1990. They were fixed in methacarn (6:3:1 methanol–chloroform–acetic acid) overnight at 4°C, processed according to routine methods and embedded in paraffin. Control tissue (43 cases) was obtained from breast tissue distant to the lesion site or from non-cancer-bearing breasts. Pathological characterisation was taken from overall evaluation of material used for routine diagnosis, and included an evaluation of a 4 μm section immediately adjacent to sections taken for dPCR (see below). This section confirmed the nature of the tissue used in the PCR reaction, and in addition the cellularity of each specimen was assessed subjectively for the proportion of the cancer cellular content and designated as either 1 = more than 75%, 2 = 25–75% or 3 = <25%. In some cases samples of the lesion were taken and stored frozen in liquid nitrogen for RNA analysis.

Flow cytometric analysis

Paraffin-embedded tumours were processed for DNA flow cytometry according to the method of Hedley *et al.* (1983). Briefly, two 50 μm sections were dewaxed using two changes of xylene and rehydrated. The tissue was incubated for 30 min at 37°C in 0.5% pepsin (Sigma) in 0.9% saline adjusted to pH 1.5 with 2 N hydrochloric acid. The isolated nuclei were counted and analysed using an EPICS C flow cytometer (Coulter Electronics, Hileah, FL, USA), after staining with 0.1% propidium iodide containing 0.004% RNAse. Ten thousand nuclei were counted at 480 nm excitation and the coefficient of variation calculated using STATPACK software (Coulter Electronics).

Ploidy was assessed as either diploid (DNA index, DI, between 0.9 and 1.10) or aneuploid (DI > 1.10 and <1.90 or >2.10). Tetraploids were classified as DI between 1.90 and 2.10 with more than 20% of the cells apparently in G_2 plus M phase of the cell cycle. For inclusion the coefficient of variation for the peak value had to be less than 8%.

Cell lines and culture conditions

Human breast cancer cell lines known to have an amplification of c-*erb*B-2 were used to calibrate the relationship between differential PCR ratio values and gene copy number. The epithelial cell line 21MT2 was obtained from R. Sager (Dana-Farber Cancer Institute, Boston, MA, USA) and contains a 40-fold increase of the c-*erb*B-2 gene (Band *et al.*, 1989). The cell line UISO BCA1 was obtained from R.R. Mehta (University of Illinois, Chicago, IL, USA) and contains a 10-fold increase in the c-*erb*B-2 gene (Sasi *et al.*, 1991).

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Each cell line was grown at 37°C in air with 5% carbon dioxide added. 21MT2 was cultured in alpha minimum essential medium (MEM) (Gibco) containing 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 1 $\mu g\,ml^{-1}$ insulin, 2.8 μM hydrocortisone 12.5 $ng\,ml^{-1}$ epidermal growth factor and 10 mM HEPES. UISO BCA1 was cultured in Glasgow's minimum essential medium (GMEM) (Gibco), 10% fetal calf serum and 2 mM L-glutamine. For calibration experiments, DNA was prepared from each cell line (Sambrook *et al.*, 1989) and was mixed with control DNA derived from normal placenta (p258, one c-*erb*-B-2 gene copy), in proportions which gave a series of known c-*erb*B-2 copy numbers. The 21MT2 DNA was diluted to give c-*erb*B-2 copy numbers of 32, 24, 16, and 8, and UISO BCA1 was diluted to give copy numbers of 9, 6, 5, 4 and 3.

Immunohistochemistry

Overexpression of c-*erb*B-2 was ascertained using the rabbit polyclonal antibody, 21N, to the c-*erb*B-2 protein (Gullick *et al.*, 1987). Four micron sections of fixed tissue were dried at 56°C then stained in a three-stage peroxidase–antiperoxidase technique (Sternberger, 1986). The primary antibody, 21N, was used at a concentration of 3.3 $\mu g\,ml^{-1}$ in 0.1 M Tris-buffered saline (pH 7.6) containing 5% normal swine serum. Each section was incubated at room temperature for 90 min. Endogenous peroxidase was blocked by exposure to 1% hydrogen peroxide in methanol for 30 min before staining. Overexpression of c-*erb*B-2 was defined as the presence of brown staining of surface membrane of cancer cells. To score positive, more than 10% of cells had to show moderate to strong staining. Controls included a known positive case and a negative control employing a preincubation of the antibody with its corresponding peptide (1 $mg\,ml^{-1}$).

mRNA

Messenger RNA was extracted from frozen tumour samples and analysed by Northern blot (Thompson *et al.*, 1990). Twenty micrograms of total RNA was denatured with formamide and formaldehyde at 55°C for 20 min and RNA species separated by electrophoresis on a 1.1% agarose gel. The RNA was transferred to a nylon filter (hybond-N, Amersham, UK) by capillary action using 10 × SSC and covalently fixed to the membrane using a UV transilluminator. To detect c-*erb*B-2 mRNA the filters were hybridised with λ107, a 1.7 kb fragment of v-*erb*B-2 (Semba *et al.*, 1985), according to the method of Church and Gilbert (1984), washed to remove non-specifically attached probe and exposed to preflashed Kodak XAR film at −70°C. Filters were stripped and reprobed with α-actin (Minty *et al.*, 1981) as an internal control for loading. The extent of hybridisation of radiolabelled probe to the mRNA species was determined using densitometry and expressed with respect to hybridisation to the actin probe.

Primers and the polymerase chain reaction

Primers used in the differential PCR are listed in Table I. They were DNA sequences specific for interferon gamma (IFN-γ150), c-*erb*B-2 and interferon beta (IFN-β). The single-copy reference sequence was the 150 bp sequence from the IFN-γ gene. For dPCR four 10-μm sections of fixed paraffin-embedded tissue were added to 100 μl of lysis buffer (50 mM Tris–HCl, pH 8.4, 1 mM EDTA, 0.5% Tween 20) and boiled for 8 min (Hubbard & Anderson, 1993). Differential PCR was performed on a Techne PHC3 thermal cycler incorporating 5 μl of prepared lysed paraffin section or 200 ng of extracted DNA, 0.25 μM each primer (except for primers for IFN-β, 0.125 μM), 200 mM dNTPs (Pharmacia), × 1 *Taq* polymerase buffer (Northumbria Biotechnology Limited, NBL), 1 unit of *Taq* polymerase (NBL) and 3 μCi of [^{32}P]CTP (New England Nuclear). Cycling parameters were one cycle of 94°C for 5 min, 50°C for 1 min, 70°C for 1 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, 70°C for 1 min, and one cycle of 94°C for 1 min, 50°C for 1 min, 70°C for 5 min. Duplicate PCR products were separated by size on 2% agarose gels, and stained with ethidium bromide. Visible bands were excised, finely chopped and added to 5 ml of Optiphase-safe scintillation fluid and radioactivity present assessed as counts per minute (c.p.m.) on a Beckman scintillation counter. A correction factor was applied to compensate for the differences in numbers of CTP bases between reference and test gene. All specimens were assessed in duplicate experiments.

The results from dPCR are expressed as ratio values and were calculated by averaging the c.p.m. from duplicate gel tracks and subtracting the average experimental blank. For c-*erb*B-2 a correction factor of 1.25 was applied to compensate for differences in dCTP content between IFN-γ150 (69 C bases) and c-*erb*B-2 (55 C bases). To ascertain the relative quantity of c-*erb*B-2 gene with respect to the reference gene, the corrected average c.p.m. for c-*erb*B-2 was divided by the average c.p.m. for IFN-γ150, giving in each case a result expressed as a ratio value. Similar correction factors were calculated and applied to dPCR involving amplification of IFN-β.

Results

Validation of differential PCR method

Calibration of ratio values Differential PCR was assessed for sensitivity and reproducibility in determining gene copy number. As defined here, 'one gene copy' corresponds to the normal diploid content of one cell. The results of differential PCR on the various DNA solutions of p258 and cell lines 21MT2 and UISO BCA1 with known c-*erb*B-2 gene copy number, using primers for IFN-γ150 and c-*erb*B-2, are shown in Figure 1. A comparison of the known copy numbers in each cell line sample and differential PCR ratio values showed that increasing gene copy number resulted in increasing ratio values; mean values for 1 and 40 gene copy numbers were 1.66 and 11.46 respectively. While comparison of the ratio values obtained for given copy numbers shows some variation between experiments, there was a consistent increment in this value within each experiment. Samples with large amplifications (>32) showed increased variation between duplicate tests. For the purposes of standardisation it was considered best to work on a mean value for these

Table I DNA sequences of primers used in differential PCR

Gene	*Sequence*	*Reference*
c-*erb*B-2		
Sense	5′-CCT CTG ACG TCC ATC ATC TC-3′	Frye *et al.* (1989)
Antisense	5′-ATC TTC TGC TGC CGT CGC TT-3′	
IFN-γ150		
Sense	5′-TCT TTT CTT TCC CGA TAG GT-3′	Frye *et al.* (1989)
Antisense	5′-CTG GGA TGC TCT TCG ACC TC-3′	
IFN-β		
Sense	5′-GTG TCT CCT CCA AAT TGC TC-3′	Neubauer *et al.* (1992)
Antisense	5′-GCC ACA GGA GCT TCT GAC AC-3′	

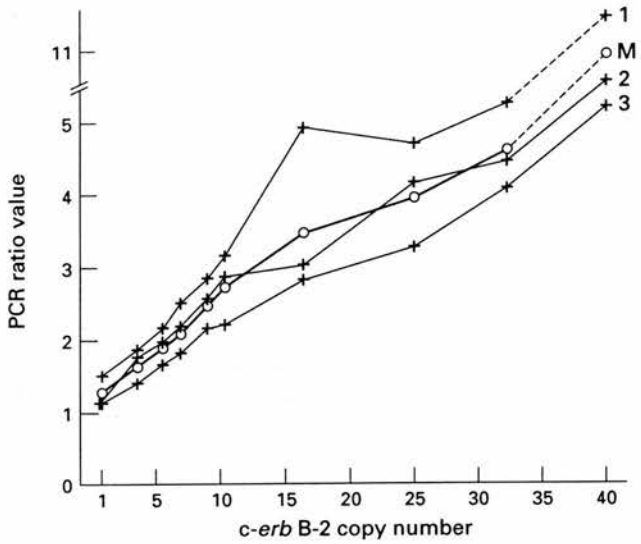

Figure 1 Relationship between c-*erb*B-2 copy number and differential PCR ratio value. Each copy number was derived by dilution of DNA from c-*erb*B-2 amplified cell lines 21MT2 or UISO BCA1 with placental DNA (one copy). Each point on line M represents the mean of triplicate experiments. Individual experiments are represented by lines 1, 2 and 3.

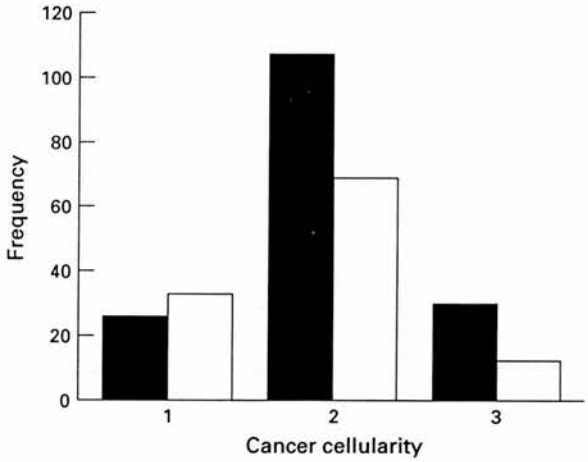

Figure 2 Relationship between cancer cellularity and frequency of c-*erb*B-2 amplification in 277 breast cancers. Cancer cellularity was assessed visually as >75% cancer cells = 1, 25–75% cancer cells = 2, <25% = 3. □, Specimens with a dPCR ratio value less than 2; ■, Specimens considered to be amplified (dPCR ratio value of 2 or above).

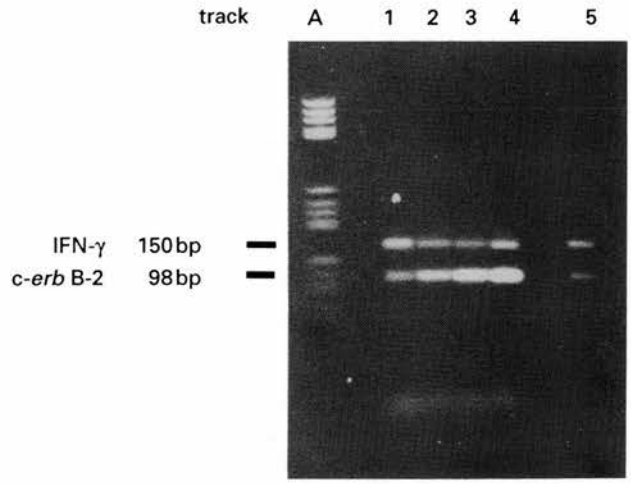

Figure 3 Differential PCR products from IFN-γ150 (150 bp) and c-*erb*B-2 (98 bp) size separated on a 2% agarose gel. Lanes are: A, BRL molecular weight marker V; 1, 2 and 3, three different cancers; 4, DNA from the same cancer as lane 3; 5, normal control DNA (p258). Differential PCR ratio values for lanes 1–5 are 1.3, 2.1, 3.5, 3.6 and 1.1 respectively. Lanes 2, 3 and 4 all show clear amplification of c-*erb*B-2 product.

experiments, depicted by open circles in Figure 1. Thus a mean ratio value of 2 approximated to five copies of c-*erb*B-2. Note that each ratio value is a derived value and does not equate with but is directly proportional to the copy number.

Factors affecting dPCR ratio values Application of this technique in a series of paraffin-embedded specimens required stringent controls. Confirmation that IFN-γ was present as a single-copy gene was obtained in 57 cancer and 27 control specimens by performing differential PCR with primers for both IFN-γ150 and IFN-β. The ranges of ratio values detected were similar for cancers (0.81–1.9) and control tissues (0.4–1.7), suggesting that IFN-γ was present in both as a single-copy gene.

Satisfactory analysis of DNA ploidy by flow cytometry was obtained from 240 cancers. In 117 the phenotype was diploid, and 123 were aneuploid or tetraploid. The frequency of amplification of c-*erb*B-2 in specimens assessed by flow cytometry was found to be highest in cancers which were diploid (60%), with lower percentages of aneuploid (47%) and tetraploid (42%) cancers being amplified. These differences were not significant.

A third potentially confounding factor was the dilutional effect of normal cells present within the cancer tissue, perhaps reducing the detection frequency of amplification. The proportion of amplified and non-amplified cases of invasive cancer ranked according to section cancer cellularity is shown in Figure 2. Amplification was found in each of the groups, including those specimens in which cancer cells constituted less than 25% of total cellularity.

c-erb*B-2 amplification and overexpression in breast cancers*

Gene amplification determined by differential PCR A total of 323 breast cancer specimens and 43 controls were tested for c-*erb*B-2 amplification using primers for c-*erb*B-2 and IFN-γ150. Figure 3 shows representative PCR products obtained from fixed tissue specimens of three different cancers and one DNA extracted by routine phenol/choroform procedures from fresh tissue preserved at −70°C from one of these cancers. Differential increase of c-*erb*B-2 products indicating amplification is illustrated, with corresponding ratio values of 1.4, 2.1 and 3.6 for the fixed cancer tissue, 3.7 for DNA of specimen 3 and 1.2 for control DNA.

The ratio values obtained using primers for c-*erb*B-2 and IFN-γ150 from both normal and cancer tissues are shown in Figure 4. The ratio range for 43 normal tissues fell consistently between 0.6 and 1.9 (mean 1.2, s.d. 0.36), and therefore values of 2 or above were considered to signify gene amplification. This value corresponds to approximately five gene copies (see Figure 1), and indicates that dPCR, in its present form, is unsuitable for exact specification of those cases with low copy number (<5). The results of duplicate experiments for each clinical case showed consistency for identification of gene amplification as being of low–medium copy number (ratio value range 2–3) or high copy number (ratio greater than 3). In cancer tissues the range was 0.6–19.2 ($n = 323$), indicating copy numbers encompassed by the range of copies assessed in the calibration (from 1 to 40). A total of 183 cancers had ratio values of 2 or above, signifying gene amplification in at least 57% of this study set. For 287 invasive cancers, the ratio values ranged from 0.6 to 19.2 with ratio values ⩾2 in 159 (55%), corresponding to low–medium copy number in 99 (34.5%) and high copy number in 60 (20.5%). For 36 *in situ* cancers the ratio ranged

from 1.0 to 8 with ratio values $\geqslant 2$ in 24 (66%), of which 15 (42%) were low–medium and nine (25%) were high copy number.

Protein overexpression assessed by immunohistochemistry Immunohistochemistry for c-*erb*B-2 overexpression was performed on 336 breast cancer specimens. Overexpression of c-*erb*B-2 was detected in 23 of 54 (43%) of *in situ* carcinomas and in 31 of 282 (11%) invasive carcinomas. In cases in which *in situ* and invasive forms of cancer were present on the same slide, no detectable differences in the staining pattern between them was observed. Staining was concentrated on epithelial cell membranes and stained cells were present evenly throughout the cancer, except in one cancer in which focal staining of cancer cells was observed.

Overexpression was not observed in normal epithelial or stromal cells.

Comparative evaluation of protein overexpression and gene amplification A case comparison of gene amplification determined by dPCR with protein expression determined by immunohistochemistry is shown in Table II. Thirty-nine of 49 immunopositive cases (80%) had gene amplification (with ratio values ranging from 2.0 to 19.2). There were ten cases in which differential PCR did not detect gene amplification in the presence of protein overexpression. However 146 of 274 immunonegative cases (53%) had PCR-detectable amplification of the c-*erb*B-2 gene, and this included 43 cases with ratio values >3, indicating high copy number. The range of differential PCR values was similar between the immunopositive and immunonegative groups (Figure 5) and applied to both *in situ* and invasive cancers. Of the 13 samples assessed by immunohistochemistry but not available for PCR, five were immunopositive.

mRNA measurement Specific messenger RNA was measured in 26 breast cancer cases, and increased levels of c-*erb*B-2 mRNA corresponding to densitometry values four times control or greater were found in 11 cases (42%). The correlation between c-*erb*B-2 mRNA levels and gene amplification and overexpression is shown in Table III. All cases with positive immunohistochemistry contained elevated levels of c-*erb*B-2 mRNA. Furthermore, 4 of 19 cases negative for immunohistochemistry also had elevated levels of mRNA; gene amplification determined by dPCR was present in two of these cases.

Discussion

This study with fixed paraffin-embedded tissue has demonstrated that dPCR is a highly sensitive technique for the detection of gene amplification and is also sufficiently robust to be applied to tumours of differing cellularity and DNA ploidy. For invasive cancers the frequency of gene amplification (55%) was considerably higher than anticipated from reports of conventional methods based on Southern or dot blotting techniques. In ten major studies of breast cancer, each assessing 100 or more cancer cases, the frequency of amplification varied between 17% and 23% (see review by Singleton & Strickler, 1992). Because of the size of the disparity some initial comment on comparability of methods is appropriate.

Study of gene amplification is complicated by terminology for an increased gene number, which may be expressed as either a fold difference, increased copy number or both; fold difference is equated with copy number in some reports (Ali *et al.*, 1988; Garcia *et al.*, 1989). We have assumed that the fold differences ascertained for the cell lines used in calibration of the dPCR are valid reflections of gene copy number, and have therefore expressed the altered dPCR ratio values as increased gene copy number. Owing to the arbitrary cut-off point for 'amplification' outwith the range observed in normals, dPCR would appear to lack the specificity to identify low copy number. Caution must be applied when ratio values are extrapolated to gene copy number in clinical cases. Experimental variation and approximations inherent to DNA analysis, including Southern or dot blotting techniques, may affect the precise relationship between classifications. Yet studies using Southern or dot blotting claim to detect increases as low as 2-fold without quoting the full range of values observed, the experimental variation in duplicate tests

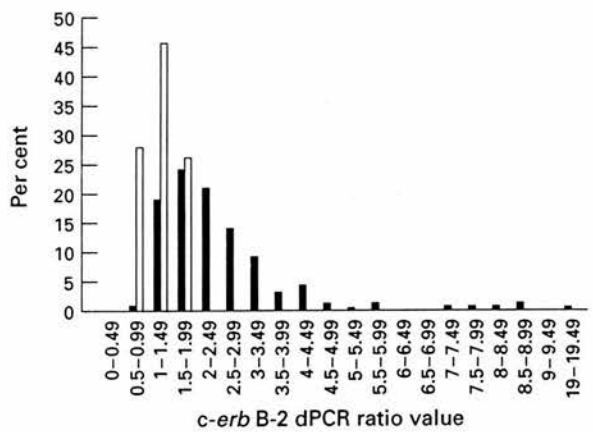

Figure 4 Distribution of differential PCR ratio values for c-*erb*B-2 in 323 cancer tissues and 43 normal control tissues. Figures in columns are expressed as percentage of cancers (■) or percentage of normal controls (□).

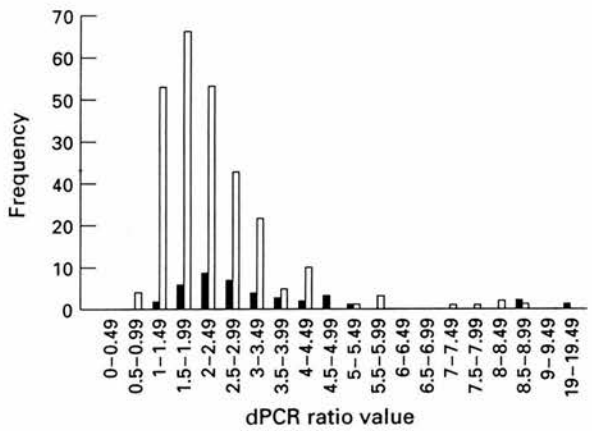

Figure 5 Distribution of dPCR ratio values for immunohistochemistry positive (■) and negative cancers (□).

Table II Comparison of c-*erb*B-2 protein overexpression measured by immunohistochemistry and c-*erb*B-2 gene amplification measured by dPCR in 323 breast cancers

	Overexpression	*No overexpression*
Amplification	39	146
No amplification	10	128

The overall frequency of amplification is 57% and overexpression 15%.

Table III Association of increased c-*erb*B-2 mRNA expression with protein overexpression and gene amplification

	Immunohistochemistry			
	Positive		*Negative*	
mRNA	Positive	Negative	Positive	Negative
Amplified	4	0	2	10
Non-amplified	3	0	2	5

All cancers with protein overexpression show elevated levels of mRNA. Four of 19 cases which were negative for protein overexpression have increased mRNA. Messenger RNA level appears to be independent of gene amplification.

or recorded cancer cellularity differences. It is of interest that a large proportion of amplified cases show a low increase in gene copy number by all techniques; for example, 44% have 2–5 copies on Southern blotting (Borg *et al.*, 1990), while in this study 61% have ratio values of 2–3. There remains some uncertainty about the most appropriate cut-off point on which to base an amplified finding, but for the purposes of this evaluation a ratio value of 2 was chosen, as this was always above the values obtained for control samples. Raising the cut-off point to a ratio value of 2.5 would reduce the numbers amplified to levels equivalent to those previously reported. However, differences in amplification frequency depending on technique have also been observed in studies of the ovary. dPCR detected c-*erb*B-2 amplification in 40% of cancers (Hruza *et al.*, 1993); in contrast, previous studies by Southern blotting detected amplification in 1–26% of ovarian cancers (Slamon *et al.*, 1989; Zang *et al.*, 1989; Imyanitov *et al.*, 1992). This suggests that there may be differences in sensitivity between these techniques. The possibility of artefactual elevation of dPCR ratio values in fixed tissue extracts was examined by comparing them with samples of DNA from the corresponding fresh tissue in a subset of cases, but we found no evidence for this (data not shown). There is also the issue of selection bias towards larger size of cancer where there is a requirement to submit tissue for extraction in DNA analysis. This does not apply to dPCR studies which, as in the present series, can be applied in a consecutive manner.

A higher degree of sensitivity than in the present study was claimed in a previous investigation of c-*erb*B-2 amplification using dPCR (Frye *et al.*, 1989). One extra copy (2-fold increase) was detectable, but that study used high-quality, homogeneous DNA derived from cell lines in a single experiment. Further developments of the technique on clinical material classified amplification in terms of fold differences, the most sensitive level detecting a 2- to 4-fold increase in c-*erb*B-2 product (Liu *et al.*, 1992; Neubauer *et al.*, 1992). Those studies used a complex algorithm of experimental exclusions involving four different dPCR reactions resulting in a selected population of cancers, and detected c-*erb*B-2 amplification in 48% of *in situ* cancers and in 21% of invasive cancers (Liu *et al.*, 1992). Details of interexperimental variation, ranges of dPCR ratio values and criteria for exclusion at each step of the algorithm were not stated. This makes direct comparison of amplification frequencies with the current study difficult. In addition Liu *et al.* (1992) restricted their series to stage II node-negative disease, whereas the present series was a consecutive group of operable cancer including both node-positive and node-negative cases. However, despite the problems of comparability, we consider that the technique as currently applied has major potential to give a valid but different perspective of gene disregulation relevant to study of the development and progression of cancer.

Detection of overexpression of c-*erb*B-2 by immunohistochemistry is subject to considerable variation between studies (Singleton & Strickler, 1993) in part because of the different primary antibodies, fixation methods, study set composition and criteria for assessing positive staining. The dilution used in this study of antibody 21N has been calibrated as detecting around 12 or more copies of c-*erb*B-2 (Gusterson *et al.*, 1988), therefore cases with an amplification of between five and 12 copies may appear to be immunonegative. Evidence from the present mRNA studies supports this limitation to detecting expression as 21% of our immunonegative cases tested had increased mRNA levels. That changes in methods can affect the frequency of detection is evident from a recent report by Poller *et al.* (1992), in which modification of fixation and immunohistochemical techniques increased the proportion of invasive cancers with c-*erb*B-2 overexpression to 39.7% from 15% (Lovekin *et al.*, 1991). As in other studies, we found good correlation between overexpression and amplification: 80% of immunopositive cancers had detectable gene amplification. However, the ranges of gene copy values found by dPCR in immunopositive and immunonegative cancers of the current study set indicate that for both invasive and *in situ* cancers amplification does not necessarily mean an equivalent overexpression, and some cases with strong immunostaining showed normal or modest increases in gene copy numbers. This suggests that factors which cause overexpression of c-*erb*B-2 in the absence of gene amplication may also play a role when gene amplification is present.

The disparity in frequency of c-*erb*B-2 gene activation between *in situ* (around 44%) and invasive cancer (around 22%) noted in previous studies (see review by Singleton & Strickler, 1992) is considerably diminished in the present analysis, but the implications for relevance in cancer progression are uncertain. An evaluation to test a hypothesis of cancer natural history in the breast by Allred *et al.* (1992) commented on c-*erb*B-2 overexpression in selected groups of 45 hyperplastic and dysplastic lesions as well as 708 *in situ* and invasive cancers. They concluded that abnormal activation of the gene was likely to be a significant but not the sole initiating factor for many cancers. The limitations of simple immunohistochemistry as a measure of disregulated gene activity have been recognised (Anderson, 1992; Wynford-Thomas, 1992). Improved sensitivity of detecting abnormal gene activity through fluorescent (or other methods of) *in situ* hybridisation (Kallionemi *et al.*, 1992; Smith *et al.*, 1993) is likely to reveal considerably more about the heterogeneity and degree of gene disregulation within cell populations. The potential to explore mechanisms of gene control by further analysis of material selected according to results of dPCR, Northern analysis and/or immunohistochemistry is however apparent from the results reported here.

The present studies show that amplification of c-*erb*B-2 is a frequent event in breast cancer and that the relationship between gene amplification and overexpression may be complex. Although the insensitivity of current immunohistochemistry in detecting small increases in protein is a factor complicating interpretation, it is nevertheless likely that each part of the replication/transcription/translation process can be disregulated. Thus combininations of such events could account for the distribution of cases among the categories of Table III. Indeed, it appears that the frequency of these various disorders of gene number and expression is not equivalent. A small percentage of cases overexpress c-*erb*B-2 in the absence of amplification, while a larger number fail to show a detectable overexpression of the gene in the presence of amplification even though a small number of this group also have increased mRNA levels. Factors acting as promoters or suppressors of gene function may directly affect transcription regardless of the amplification status. Further direct evidence of factors affecting transcription comes from studies of breast cancer cell lines in which c-*erb*B-2 protein can be down-regulated by oestrogen complexed with its receptor (Russell and Hung, 1992). Increased c-*erb*B-2 mRNA levels resulting from elevated amounts of a transcription factor have also been observed in cancer cell lines which have no detectable gene amplification (Hollywood & Hurst, 1993). Other explanations of disorder include physical damage to the gene, mutation or the absence of promoters. This variety of biological events and consequences suggest that a more realistic model to evaluate c-*erb*B-2 disregulation in breast cancer must encompass a greater number of circumstances and consider the interaction of other biological processes. The potential to study these in subgroups of suitably characterised breast cancer cases is apparent.

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THE BREAST

Methodology

Simple 10 min preparation of fixed, embedded breast tissue for the polymerase chain reaction

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SUMMARY. **A speedy method of preparing archival material for polymerase chain reaction studies is presented.**

INTRODUCTION

Analysis of putative oncogenes or tumour supressor genes within breast cancers has recently been greatly simplified by the polymerase chain reaction (PCR). However sample preparation can be a major time consuming component of test amplifications with the PCR. We have therefore developed a rapid method of sample preparation from fixed tissues, including those embedded in paraffin, which gives reproducible, high quality amplification of single copy gene markers by PCR, even from small breast specimens. It is economical with valuable historical material and the fewer stages of manipulation leads to fewer opportunities for specimen contamination. A wide variety of preparative methods for PCR from fixed material have been described previously[1, 2] but comparative drawbacks include lengthy preparation and incubation steps or an excessive requirement for source material.

Methodology

We obtained breast tissue removed at routine surgical operations and fixed either in methacarn (6:3:1 methanol, chloroform, acetic acid) or 10% buffered formalin. The fixed tissues were each routinely processed and embedded in paraffin. Prior to sectioning, excess fat was trimmed and then ten micron sections were cut on a Reichert-Jung 2030 microtome and transferred to separate 1.5ml eppendorf tubes. The knife was cleaned with xylene between each specimen. 100µl of lysis buffer (50mM Tris pH 8.4, 1mM EDTA, 0.5% Tween 20) was added and the specimen was boiled for 8 minutes in a heated water bath. The resulting tissue DNA solution was used either directly or stored frozen at –20°C. For control purposes, DNA was prepared from fresh breast tissue, taken at surgery from the same specimens at adjacent sites, according to routine extraction methods.[3]

Amplification of DNA from each solution was performed on a Techne PHC3 thermal cycler using 5µl of the prepared specimen, 200 µM dNTP, 250 pM each of sense and antisense primers, 1 unit of Taq polymerase (NBL), 5µl of x10 reaction buffer (NBL) to a total volume of 50µl. Using primers for c-erb B2 and interferon gamma (IFNG),[4] 30 cycles were performed at their respective optimal temperatures. We tested four other primer sets developed for detection of polymorphic sites in the human genome.

Results of amplification procedures are shown in the figure. The specificity of the PCR reaction for c-erb B2 and IFNG in breast specimens is the same for DNA, formalin and methacarn fixed specimens. Apparent differences in band intensities are probably due to differences in the quantity of template added to the initial PCR reaction. In general, it appeared that formalin fixed tissue gave a weaker signal than methacarn fixed tissue in comparable blocks (data not shown), therefore it is possible that methacarn fixation is less deleterious to tissue DNA than formalin. DNA, including that from single copy genes (IFNG) was successfully amplified using six different primer sets, the longest sequence of amplified DNA was 400bp. The upper limits of amplicon size using this preparative technique have not been established. We have successfully analysed over 300 specimens using this preparative technique including normal breast tissue, breast cancer and lymph node. Detectable PCR product has been obtained from as little as one 5 micron section although the amount of tissue

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Fig—PCR amplified DNA from control DNA (D), methacarn fixed (M), and 10% buffered formalin fixed (F) tissue. The amplimers used were c-erb B2 (tracks 1–3) and IFNG (tracks 4–6). The samples were electrophoresed on a 2% agarose gel and stained in ethidium bromide.

required is likely to depend on the sectional area and cellularity of each specimen. Prepared specimens can be successfully stored at –20°C, and undergo at least 8 freeze-thaw cycles without diminution of amplified product. This is of great advantage where multiple tests are required on each specimen.

COMMENT

Preparation of PCR template from cell lines and peripheral blood leucocytes commonly employ a non-ionic detergent incubation followed by digestion with proteinase K. However when preparing template DNA from fixed embedded tissue the process has been extended by dewaxing and a lengthy (such as 5 days) incubation in proteinase K. Also specimens prepared using proteinase K, without subsequent DNA extraction, can be unstable after freezing. This method substitutes boiling in detergent for proteinase K digestion and DNA extraction. The present findings suggest that this method releases sufficient DNA from the tissue for successful PCR and that irrelevant protein which may also be released does not greatly affect the reaction. Despite recent suggestions by Doyle and O'Leary[5] that fixation methods may play a role, we found that the subsequent preparative technique was of as great a significance in successful PCR.

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Disregulation of Urokinase Plasminogen Activator Gene in Breast Cancer

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Disregulation of urokinase plasminogen activator (uPA) was assessed in 134 breast cancer specimens. Overexpression of uPA was determined by immunohistochemistry using the specific monoclonal antibody, #394. Gene amplification was assessed by differential polymerase chain reaction, using primers designed to amplify a 111 bp segment of the uPA gene. Overexpression of uPA was detected in 33% of breast cancers, including 4 of 21 *in situ* carcinomas, 7 of 14 lobular and 2 of 10 tubular carcinomas. Overexpression of uPA did not correlate with the presence or absence of oestrogen receptors. uPA gene amplification was not detected in any cancer. We conclude that uPA gene amplification is not a major mechanism instigating uPA overexpression in breast cancer, and that overexpression is likely to be controlled by other mechanisms.

Key words: breast carcinoma, urokinase plasminogen activator, gene amplification, gene overexpression
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INTRODUCTION

OVEREXPRESSION OF the serine protease,urokinase plasminogen activator (uPA) in epithelial cells has been linked to aggressive behaviour in breast cancer [1, 2]. The biochemical action of the enzyme may promote metastasis by degradation of proteins, such as collagen, in the extracellular tumour stroma [3]. The conversion of plasminogen to plasmin by uPA is thought to be a critical step in this degradation [4]. Previous studies of breast cancers with immunohistochemistry using a monoclonal antibody directed against human uPA have shown overexpression

of uPA in all of 115 cancers tested [1]. Analysis of uPA antigen by ELISA in the cytosolic fraction of these cancers indicated that they contained, on average, 11 times more uPA than normal breast tissue [1]. High concentrations of uPA in breast cancer tissue may be an independent prognostic factor in predicting early relapse [1, 5, 6, 7].

The mechanisms of overexpression of uPA in breast cancer are not clear, and may involve disregulated promoter and/or suppressor activity, or fundamental DNA changes such as gene amplification. The regulation of the concentration of uPA in the cytoplasm of cells is complex; it can be influenced by a number of cell signals such as hormones [8], phorbol esters [9], growth factors [10], and cytokines [11]. Another possible mechanism for overexpression is gene amplification. Overexpression associated with gene amplification has been demonstrated in breast cancer for some oncogenes, such as c-*erb* B2 and c-*myc* [12–14], and have shown that these genes can be disregulated by more than one mechanism. We examined the possibility that overexpression of uPA could be due, in some cases, to uPA gene amplification.

MATERIALS AND METHODS

Breast tissues

Samples of tissue were collected from 134 breast cancers at routine surgical operations, which included mastectomy and open biopsy for both palpable and non-palpable lesions; all were primary cancers which had not been included in neo-adjuvant treatment studies [15]. Samples were restricted to the age group 50–65 years and were collected from January 1988 to May 1990. They were fixed in methacarn (6 : 3 : 1, methanol : choloroform : acetic acid) overnight at 4°C, processed according to routine methods and embedded in paraffin. Control tissue was obtained from breast tissue distant from the lesion site or from non-cancer bearing breasts. Pathological characterisation was taken from overall evaluation of material used for routine diagnosis and assessed according to standard criteria for histological type, grade, and node status [16]. This included an evaluation of a 4 μm section immediately adjacent to the tissue used for differential PCR (see below) to confirm its histopathological nature. The specimen's cellularity, namely the proportion of cancer cells present, and ploidy status was assessed according to published methods [17].

Oestrogen receptors

Oestrogen receptors were measured from tissue collected, frozen and stored at −196°C at the time of operation. A radioligand-binding assay using dextran-coated charcoal was used according to the method of Hawkins and colleagues [18]. The concentration of oestrogen receptors in each cancer sample was expressed as a concentration of protein, with values of >20 fmol/mg protein considered clinically significant.

Immunohistochemistry

Expression of uPA protein was examined with a murine monoclonal antibody, #394, claimed as specific for human urokinase plasminogen activator (American Diagnostica Inc. New York, NY, U.S.A.). Four micron paraffin sections of all cancers were dried at 56°C overnight. The sections were dewaxed in xylene and rehydrated to water. Endogenous peroxidase was blocked by exposure to 1% hydrogen peroxide in methanol for 15 min before staining. The sections were washed in Tris-buffered saline (TBS pH 7.6), followed by a 10 min incubation with normal rabbit serum (Scottish Antibody Production Unit, Carluke, U.K.) diluted 1 : 5 with TBS. The primary antibody was applied at a 1 : 100 dilution in normal rabbit serum/TBS and incubated at room temperature for 30 min. Normal rabbit serum was used in place of the primary antibody as a negative control. An ABComplex method was carried out according to manufacturers instructions (Dako K355). The sections were lightly counterstained in haematoxylin. To score positive, indicating uPA expression, cancer cells had to show weak, moderate or strong brown staining of cytoplasm above normal stromal components of the tissue section, excluding macrophages. The assessment of uPA expression was based on a single tissue section from each specimen and was jointly assessed by TJA and ALH. A positive control, a cancer previously identified as showing strong staining with #394, and a negative control were incorporated in each set of sections for staining. Some variation in staining intensity was noted in positive control sections. As the titre of antibody #394 dropped from 1 : 100 to 1 : 10 during overnight storage after reconstitution of the lyophilised preparation, fresh dilutions of #394 were prepared for each batch of slides. This occurred with three separate batches of antibody.

Primers and the differential polymerase chain reaction

Primers for differential PCR for uPA were designed from the human gene sequence published elsewhere [19]. The primer sequence for the sense strand was 5′ CAGTTTACCCTCACCCTGGA 3′ (1631–1650 bp), and the antisense strand 5′ AGCCAACTGTTGTAGGGGTG 3′ (1757–1738 bp). PCR using these primers yields a 111 bp product from the intron 2-exon 3 boundary of the published sequence. The single copy reference gene used in dPCR was a 150 bp sequence of human interferon gamma [20]. For dPCR, four 10 μm sections of fixed, paraffin-embedded tissue were added to 100 μl of lysis buffer (50 mM Tris HCl, pH 8.4, 1 mM EDTA, 0.5% Tween 20) and boiled for 8 min [21]. Differential PCR was performed on a Techne PHC 3 thermal cycler incorporating 5 μl of prepared lysed paraffin section or 200 ng of control DNA (human placental), 0.25 μM each primer, 200 mM dNTPs (Pharmacia), ×1 Taq polymerase buffer (Northumbria Biotechnology Limited NBL), 1 unit of Taq polymerase (NBL) and 3 μCi of ^{32}P CTP (New England Nuclear). Cycling parameters were one cycle of 94°C for 5 min, 50°C for 1 min, 70°C for 1 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, 70°C for 1 min and one cycle of 94°C for 1 min, 50°C for 1 min, 70°C for 5 min. Duplicate PCR products were separated by size on 2% agarose gels, and stained with ethidium bromide. Visible bands were excised, finely chopped and added to 5 ml of Optiphase-safe scintillation fluid. Radioactivity was assessed as counts per minute (CPM) on a Beckman scintillation counter. Results from dPCR are expressed as ratio values and were calculated by averaging the CPM from duplicate gel tracks and subtracting the average experimental blank. For uPA, a correction factor of 1.04 was applied to compensate for differences in dCTP content between amplicons IFNG150 (69 C bases) and uPA (66 C bases). To ascertain the relative copy number of uPA to the reference gene, the corrected average CPM for uPA was divided by the average CPM for IFNG150, giving in each case a result expressed as a ratio value.

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This method has been previously validated for use of fixed breast tissues and shown to detect amplifications of the oncogenes c-*erb* B2, EGFR and N-*ras* [17, 20].

RESULTS

Immunohistochemistry

Immunohistochemistry for expression of uPA protein was performed on 134 breast cancer specimens. Overexpression of uPA protein was observed in 40 of 113 (35%) invasive cancers and in 4 of 21 (19%) *in situ* cancers, (total cancers 33%). Staining was concentrated in the cytoplasm of the cells, and usually present evenly throughout the cancer cell population (Figure 1). Staining of the cell membrane was present in some positive cancers, however, this was always in association with cytoplasmic staining. In some cases, weak staining was observed in normal stroma and infiltrating lymphocytes, but this was always in association with positively stained cancer cells. Strong staining was observed in cells identified as macrophages in a small number of cases, and if staining was present only in macrophages, specimens were considered negative.

Table 1 summarises the association between uPA protein overexpression and features of the cancers studied. uPA expression was more common in invasive cancers, and within that group was more frequent in oestrogen receptor negative cancers. "Positive" staining was present in similar proportions in each of the ploidy groups, diploid (36%), aneuploid (43%) and tetraploid (38%). We found no correlation between size of cancer or node status with overexpression. Grade 1 cancers showed a lower frequency of overexpression (24%) than grade 2 or 3 (38%), but these differences were not significant.

Overexpression was not confined to any particular histological type of cancer, but noteworthy was the high proportion in lobular carcinomas (7 of 14), and overexpression was also present in some tubular carcinomas (2 of 10). Freqency of overexpression in each type of cancer is shown in Table 1.

Differential PCR

Figure 2 shows representative dPCR products obtained from three cancer specimens and a normal DNA control (derived from human placenta). Differential PCR ratio values were obtained for 134 cancer specimens and 33 control tissues (Table 2), and ranged from 0.52 to 1.54 (mean 0.9) in control tissues and 0.41–1.83 (mean 1.1) in cancer tissues. Ratio values of 2 or above have been considered to signify gene amplification

Table 1. Clinical features of the study population with uPA immunohistochemistry

	Number of cases	Percentage with positive immunohistochemistry
Invasive cancers	113	35
In situ cancers	21	19
Invasive cancers only		
Oestrogen receptor status		
<20 fmol/mg protein	32	41
⩾20 fmol/mg protein	73	34
NK	8	
DNA ploidy		
Diploid	45	36
Aneuploid	28	43
Tetraploid	24	38
NK	6	
Cancer size		
1–10	10	50
11–20	38	24
21–30	41	44
31–40	12	33
>40	12	33
Lymph node status		
Positive	36	31
Negative	68	38
NK	9	
Cancer grade		
1	21	24
2	66	38
3	21	38
NK	5	
Cancer type		
DCI NST	86	35
LCI	14	50
TCI	10	20
OTH	3	33

Invasive cancer types are DCI NST, ductal carcinoma of no special type; LCI, lobular carcinoma; TCI, tubular carcinoma; and OTH, other special types; NK, not known.

in other studies [17]. There was no clear difference in range of values between invasive cancers (0.41–1.83) and *in situ* cancers (0.7–1.55). The distribution of ratio values was similar for immunohistochemistry-positive and -negative cancers (Figure 3). Cancers shown to be overexpressing uPA protein were distributed evenly over the range of dPCR values, indicating that cancers which overexpress uPA are not associated with high dPCR values. Differential PCR values were not influenced by specimen cellularity or DNA ploidy.

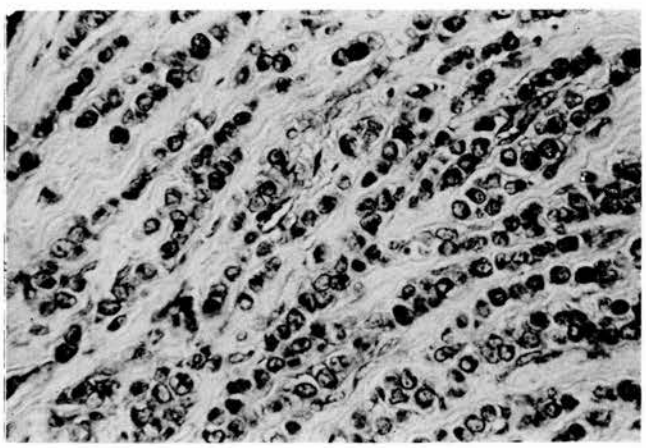

Figure 1. Four micron section of a lobular carcinoma showing strong positive staining with anti-uPA antibody (#394). Magnification × 160.

DISCUSSION

Using dPCR to study gene disregulation at the uPA locus, we found no evidence for amplification of this gene. We could not detect any relationship between overexpression of uPA protein and dPCR ratio values indicating increased gene copy number. We therefore conclude that uPA gene amplification is not a major influence on immunohistochemically detected overexpression of uPA in breast cancer. However, other interesting features were observed and are noteworthy of discussion.

Although no difference was detected between the ranges of dPCR ratio values for cancers and controls, cancers had a higher mean dPCR value. This could reflect a low level of chromosomal

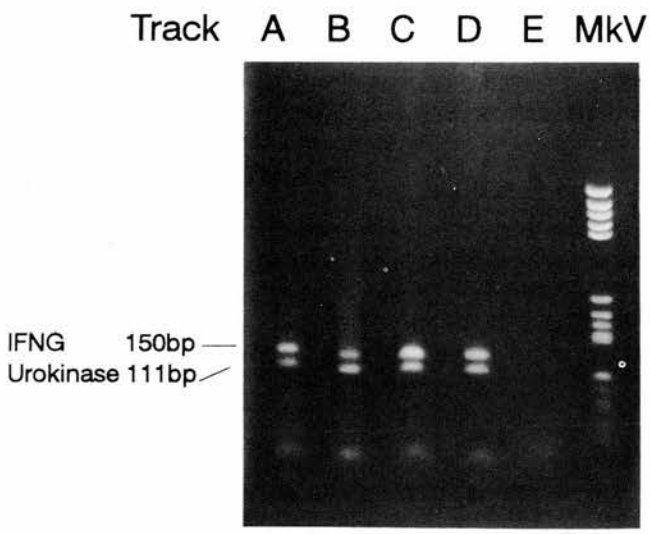

Figure 2. Differential PCR products from three different breast cancers (Tracks A, B and C) and one control placental DNA (Track D), separated on 2% 3 : 1 Nuseive/Seakem agarose gel. Track E contains the experimental negative control (PCR reaction minus template DNA). PCR products for IFNG (reference gene) and uPA are indicated at 150 bp and 111 bp, respectively.

Table 2. Ranges of dPCR ratio values found for invasive, in situ *breast cancers and control tissues*

	Number of cases	dPCR ratio range	Mean dPCR ratio value
Invasive cancers	113	0.41–1.83	1.12
In situ cancers	21	0.7 –1.55	1.18
Controls	33	0.52–1.54	0.90

instability, yet there was no association between high dPCR values and abnormalities of DNA ploidy. Furthermore, cases overexpressing uPA protein were not solely restricted to those with dPCR ratio values at the upper end of the normal range, indicating that overexpression was not due to minor chromosomal disregulation. The possibility that gene overexpression can lead to gene amplification has been suggested [22, 23], but has not been confirmed experimentally and seems unlikely to be true for the uPA gene.

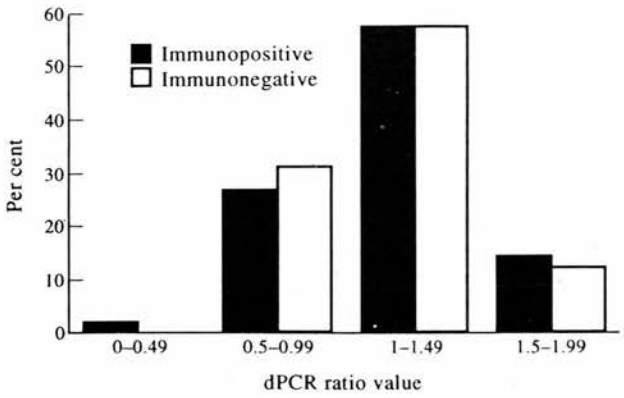

Figure 3. Range of dPCR values for immunohistochemistry positive and negative cancers.

We found uPA expression to be common in invasive lobular cancers. These cancers characteristically infiltrate through stromal elements as individual units in linear array, and uPA may play some part in this process. However, both invasive tubular carcinomas and *in situ* cancers also expressed uPA, yet these histological types are recognised for cell cohesion, differentiation and lack of metastasis [24, 25]. This suggests that overexpression of uPA can be an 'early' event and that additional factors are required for the development of invasion resulting in a poorer prognosis. The presence of uPA in the cytoplasm is probably only one of several possible factors involved in cancer cell dissemination. Examination of lung carcinoma cell lines have indicated that functionally active, receptor-bound, uPA is essential for tissue invasion [26]. Studies of uPA overexpression combined with assessment of cell surface receptors (uPAR) may illuminate the significance of overexpressing uPA, especially in cancers which have a favourable outcome.

Assessing the mechanisms accounting for overexpression of uPA in breast cancer is likely to be complex, and may involve factors which affect transcription, translation and post-transcriptional processing. Increased levels of specific uPA inhibitors, PAI-1, PAI-2 and protease nexin, in breast cancer cells have been shown to regulate cytoplasmic concentration of uPA [27]. Post-transcriptional activation mechanisms of uPA may also be disregulated in cancer cells [28]. Immunohistochemistry is a convenient method for demonstrating disregulated gene expression, and detection frequency is usually high, in the order of 88–100% of breast cancers [1, 29]. However, this study detected overexpression of uPA in only 33% of breast cancers, a frequency similar to another immunohistochemical study of breast cancer, although that study was based on frozen sections [30]. This recorded disparity in frequency of positivity is a cause for concern, but could be due to criteria for scoring positivity, differences in immunohistochemical protocols and antibodies, or both of these. Present requirement for staining above that of normal stroma was an excluding factor in only 6 cases, and is unlikely to be a major factor influencing the frequency of positivity. The instability of the antibody used here is a major factor causing concern and has been noted in other laboratories (M. Walch, personal communication) for unidentified reasons. There are alternative antibodies available for uPA which also detect high frequencies of expression in ELISA [7], but present experience suggests caution before drawing conclusions from immunohistochemistry alone.

Other features of our cancer population, such as size and ploidy, show no particular associations with uPA expression. The trends toward elevated uPA expression in grade 2 or 3 cancers and in negative lymph node status cancers were noted previously [6, 7]. Correlations of biochemical and immunochistochemical procedures generally have indicated that measured concentrations of uPA protein correlate with the strength of immunostaining, although individual cases can show major discrepancies [1]. This suggests that the relationship between immunohistochemical staining and detection of uPA protein in breast cancers is not strictly quantitative. Furthermore, the results of biochemical studies may be confounded by the presence of uPA-rich macrophages [31], indicating that a combination of immunohistochemistry and ELISA would be more informative than either technique alone. Comparability between studies is further confounded by differences in the experimental strategy and antibodies employed [1, 2, 6, 7, 30]. The concentrations of uPA in the cytoplasm of cancer cells is known to be affected by growth factors, phorbol esters, cytokines and

hormones [9–11, 32]. Studies of cell lines have shown that oestrogen complexed with its receptor can induce uPA synthesis [32]. We found no significant difference in the frequency of uPA protein overexpression in oestrogen receptor-positive and -negative cancers, suggesting that oestrogen is not a major influence in the complex regulatory mechanisms of uPA production.

In summary, our study has provided evidence that uPA is overexpressed in at least some breast cancers which may relate to gene disregulation, though not to gene amplification.

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